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MECHANISMS OF ACTIVATION OF THE LEUKOCYTE INTEGRIN LFA-1

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Life Sciences

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for the degree of Doctor of Philosophy
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DEDICATION

To Mum and Dad
for their support, encouragement and suffering
through all the years of exams the boys and I have put them through!

ABSTRACT

This work was undertaken to characterise the interaction of the leukocyte-restricted integrin LFA-1 (CD11a/CD18) with its ligands. LFA-1 function is critical for an immune response and, for example, allows leukocyte binding and transmigration across the endothelium, antigen presentation and cytotoxic T lymphocyte-mediated killing. The ligands for LFA-1 are the Intercellular Adhesion Molecules (ICAMs), with ICAM-1, ICAM-2 and ICAM-3 being the best characterised.

The binding sites on ICAM-1 and ICAM-3 for LFA-1 were investigated with the use of antibodies and mutated proteins. The following regions were found to have a role in binding LFA-1: the CFG face of ICAM-3 domain 1; domain 2 of ICAM-1; a residue in domain 1 of ICAM-1 that is mutated at high frequency in African populations and is associated with susceptibility to cerebral malaria.

Binding of Mg^{2+} or Mn^{2+} to the extracellular region of LFA-1 and intracellular signalling can both stimulate LFA-1 to adhere to ICAM-1, but by different processes. The former mechanism induces a high affinity form of LFA-1, which was shown to be achieved by an inter-domain movement involving the I domain of the LFA-1 α subunit. This is the first physical evidence for a conformational change occurring in an integrin upon activation. The mechanism by which intracellular signalling activates LFA-1 was demonstrated to involve calpain-dependent clustering of LFA-1 in the membrane, thus increasing the avidity of LFA-1 for ICAM-1.

Leukocyte Adhesion Deficiency-1 and Glanzmann's Thrombasthenia are genetic disorders in which mutations in the integrin genes result in absence of expression or expression of a non-functional integrin. The defects in function of leukocytes from a patient with clinical features of both disorders were studied. The results suggest that the patient has a novel form of integrin dysfunction in which integrins are expressed at normal levels, can be induced to bind their ligands by mechanisms which increase the affinity of interaction, but cannot be stimulated to bind ligand by intracellular signalling pathways.

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ABBREVIATIONS AND DEFINITIONS

APC	antigen presenting cell
APS	ammonium persulphate
ARF	ADP ribosylation factor
BCECF	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester
Brij 96	polyoxyethylene 10 oleyl ether
BSA	bovine serum albumin
[Ca ²⁺] _i	intracellular Ca ²⁺ concentration
CBZ-LVG	CBZ-Leu-Val-Gly diazomethyl ketone
CD	circular dichroism
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
cSMAC	central supramolecular activation cluster
C-terminal	carboxy-terminal
DAG	diacylglycerol
DC	dendritic cell
DC-SIGN	dendritic cell-specific ICAM-3 grabbing non-integrin
DEAE-dextran	Diethylaminoethyl-dextran
DHR	di-hydrorhodamine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid disodium salt
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (β-aminoethy ether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERM	ezrin/radixin/moesin
FCS	foetal calf serum
FITC	fluorescein isothiocyanate

fMLP	N-formyl-methionyl-leucyl-phenylalanine
GEF	guanine exchange factor
GMEM-S	supplemented Glasgow minimum essential medium
GST	glutathione S-transferase
HBSS	Hanks balanced salt solution
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HEV	high endothelial venules
H-HBSS	HEPES buffered HBSS
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
ICAM-1Fc	the extracellular domains of ICAM-1 fused to the Fc portion of human IgG ₁
ICAM-1 ^{Kilfi}	ICAM-1 with the Lys ²⁹ /Met polymorphism
ICRF	Imperial Cancer Research Fund
I domain	inserted/interactive domain
I-Fc	LFA-1 I domain fused to the Fc portion of human IgG ₁
I-GST	LFA-1 I domain fused to glutathione S-transferase
IFN-γ	interferon-γ
Ig	immunoglobulin
IgSF	immunoglobulin supergene family
IL-1β	interleukin-1β
IL-2	interleukin-2
IP	immunoprecipitate
IP ₃	inositol 1,4,5-trisphosphate
IPTG	isopropyl-1-thio-β-D-galactopyranoside
JAB-1	jun activation domain binding protein-1
JAM	junctional adhesion molecule
JNK	c-jun N-terminal kinase
kDa	kilo daltons
LAD-1	leukocyte adhesion deficiency-1
LFA-1	leukocyte function associated antigen-1
LIBS	ligand induced binding site
LW	Landsteiner Wiener

mAb	monoclonal antibody
MacMARCKS	macrophage-enriched myristoylated alanine-rich C kinase
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MHC	major histocompatibility complex
MIDAS	metal ion-dependent adhesion site
min	minute
MTT	3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue
m1h2-3 ICAM-3Fc	murine ICAM-1 domain 1 fused to human ICAM-3 domains 2 and 3 fused to the Fc portion of human IgG ₁
NIF	neutrophil inhibitory factor
NK	natural killer
N-terminal	amino-terminal
OPD	o-phenylenediamine dihydrochloride
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PdBu	phorbol-12,13-dibutyrate
PECAM-1	platelet endothelial cell adhesion molecule-1
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PHA	phytohaemagglutinin
PIP ₂	phosphatidylinositol 4,5,bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PSI	plexins, semaphorins and integrins
pSMAC	peripheral supramolecular activation cluster
Rack-1	Receptor for activated protein kinase C-1
RPMI 1640	Roosevelt Park Memorial Institute medium 1640
RT	room temperature
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHIP	SH2-containing inositol phosphatase
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine

TNF- α	tumour necrosis factor- α
Triton X-100	t-octylphenoxypolyethoxyethanol
Tween 20	polyoxyethylenesorbitan monolaurate
VCAM-1	vascular cell adhesion molecule-1
wt	wild type

CHAPTER 1

INTRODUCTION

1.1 THE INTEGRINS

The integrins are a widely expressed family of cell adhesion molecules which mediate cell-cell and cell-extracellular matrix (ECM) interactions. They were termed “integrins” to denote their role in integrating the extracellular environment and the cytoskeleton (Tamkun et al., 1986; Hynes, 1987). Integrins are heterodimeric integral membrane glycoproteins composed of non-covalently linked α and β subunits. Each subunit consists of a large extracellular region (approximately 1000 amino acids for the α subunit and 700 amino acids for the β subunit), a single hydrophobic transmembrane domain and a cytoplasmic tail, which is short in most subunits. To date eighteen α subunits and eight β subunits have been identified that associate to form more than twenty different $\alpha\beta$ complexes (Fig. 1.1).

The extracellular region of integrins recognises a variety of ligands including extracellular matrix proteins, cell surface Immunoglobulin Superfamily members and plasma proteins (Table 1.1). In addition, several pathogens bind to integrins. Each combination of α and β subunit defines a particular ligand binding specificity. Most integrins bind more than one ligand and, conversely, each ligand can often bind more than one integrin. Many ligands contain the motif Arg-Gly-Asp (RGD), which mediates binding to integrins. The cytoplasmic tails bind cytoskeletal and regulatory proteins and the transmembrane domains can make lateral associations with other cell surface molecules, such as members of the tetraspan family. Both these types of associations can regulate integrin activation.

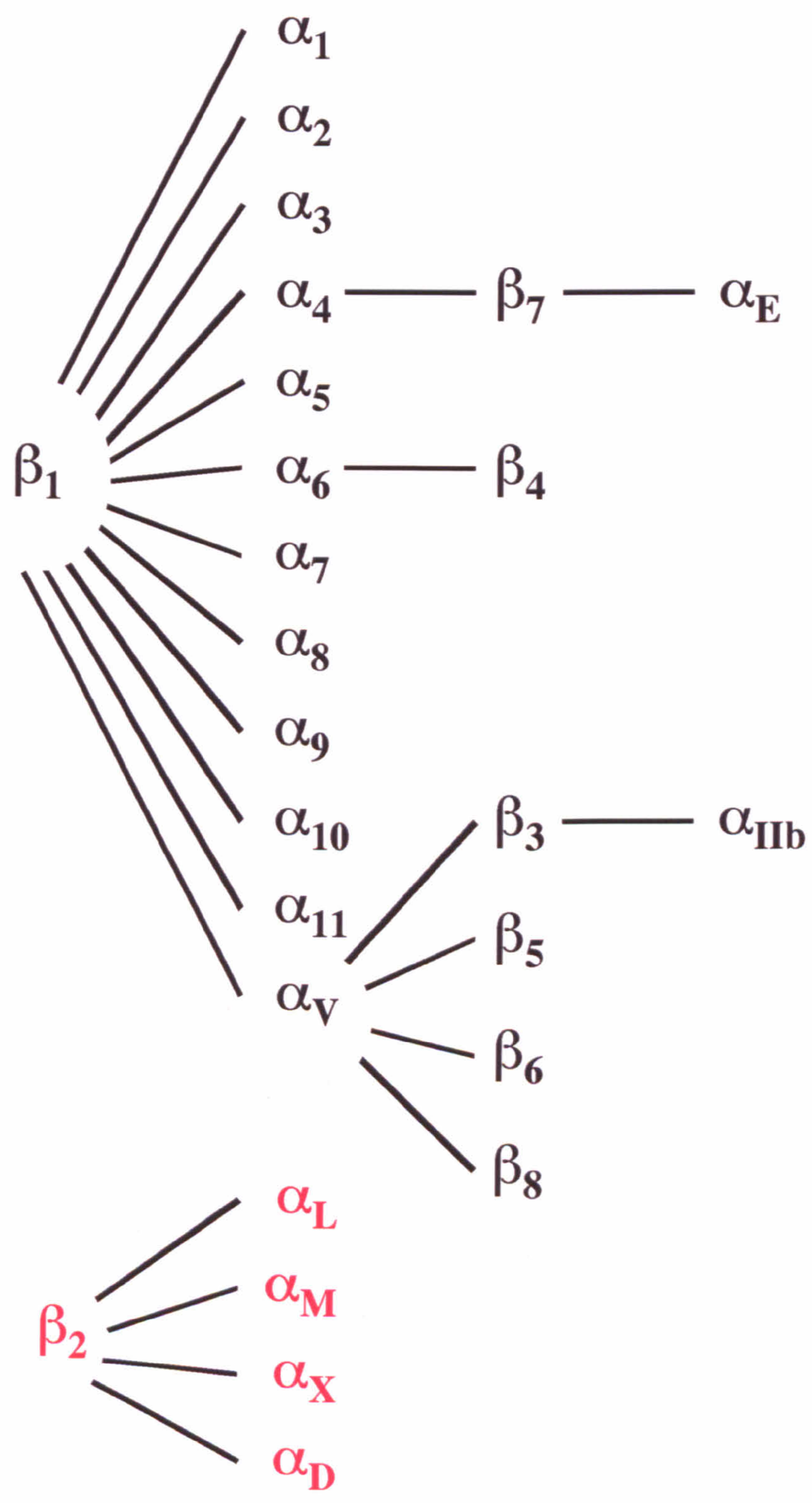


Figure 1.1 The integrin family of adhesion molecules
The subunits in red are members of the leukocyte integrin family.

INTEGRIN SUBUNITS		LIGANDS
β_1	α_1	Collagen, Laminin
	α_2	Collagen, Laminin, Echovirus 1
	α_3	Collagen, Laminin, Fibronectin, Epiligrin, Entactin, Invasin, Thrombospondin
	α_4	Fibronectin, VCAM-1, Invasin, Osteopontin
	α_5	Fibronectin, Invasin
	α_6	Laminin
	α_7	Laminin
	α_8	Fibronectin, Vitronectin, Tenascin, Osteopontin
	α_9	Tenascin, Osteopontin, VCAM-1
	α_{10}	Collagen
	α_{11}	Collagen
	α_v	Fibronectin, Vitronectin
β_2	α_L	ICAM-1, ICAM-2, ICAM-3, ICAM-4 and ICAM-5
	α_M	Fibrinogen, iC3b, Factor X, ICAM-1, ICAM-2, Elastase, Heparin, High Molecular Weight Kininogen, NIF, Denatured proteins
	α_X	Fibrinogen, iC3b, Collagen, Denatured proteins, (ICAM-1?)
	α_D	ICAM-3, VCAM-1
β_3	α_{IIIb}	Fibrinogen, Fibronectin, Thrombospondin, von Willebrand Factor, Vitronectin
	α_v	Fibrinogen, Fibronectin, Thrombospondin, von Willebrand Factor, Vitronectin, Collagen, Osteopontin, Tenascin
β_4	α_6	Laminin
β_5	α_v	Vitronectin
β_6	α_v	Fibronectin, latent TGF- β_1
β_7	α_4	MAdCAM-1, VCAM-1, Fibronectin
	α_E	E-cadherin
β_8	α_v	Vitronectin

Table 1.1 The integrins and their ligands

Divalent cations are essential for ligand binding by integrins and also for heterodimer formation. Putative cation binding sites exist in both the α and β subunits. Most integrins are constitutively inactive in resting leukocytes but are rapidly activated in a tightly regulated fashion in response to stimuli. Activation of integrins is associated with increased affinity for ligand, epitopes for certain mAbs being revealed, clustering of the integrin, and alterations in connections with the cytoskeleton (reviewed in (Arnaout, 1990; Hynes, 1992)).

1.1.1 The Leukocyte Integrins

Lymphocyte function-associated antigen 1 (LFA-1), Mac-1, p150,95 and $\alpha_D\beta_2$ are integrins which share a common β chain, β_2 (CD18), in association with the α chains α_L (CD11a), α_M (CD11b), α_X (CD11c) and α_D (CD11d), respectively (Fig. 1.1). These four integrins are referred to as the Leukocyte Integrins as their expression is restricted to leukocytes. The genes for all four leukocyte integrin α subunits are located in a cluster on chromosome 16 band p11 (Corbi et al., 1988; Wong et al., 1996), while the β_2 gene is on chromosome 22 band q21 (Marlin et al., 1986; Corbi et al., 1988). α_M , α_X and α_D share approximately 60% amino acid identity, but α_L is less homologous with 35% identity.

1.1.1.a LFA-1

LFA-1 was first discovered through characterisation of monoclonal antibodies in the early 1980s, first in mice (Kurzinger et al., 1981; Pierres et al., 1982), and a few years later in humans (Hildreth et al., 1983). LFA-1 is expressed on virtually all leukocytes and binds to the intercellular adhesion molecules (ICAM)-1 (Marlin and Springer, 1987), ICAM-2 (Staunton et al., 1989a), ICAM-3 (de Fogerolles et al., 1993; Fawcett et al., 1992; Vazeux et al., 1992), ICAM-4 (Bailly et al., 1995) and ICAM-5 (Mizuno et al., 1997; Tian et al., 1997). These interactions

play an important role in the immune response allowing, for example, proliferation of lymphocytes, cytotoxic T cell responses, natural killer (NK) cell activity, T cell-B cell interaction and leukocyte extravasation (reviewed in (Shimizu et al., 1999)).

1.1.1.b Mac-1

Mac-1 is primarily expressed on granulocytes, monocytes and NK cells. In unstimulated neutrophils only 5% of Mac-1 is expressed on the cell surface, 75% is found in granules and the remaining 20% in secretory vesicles (Sengelov et al., 1993). Upon cell activation there is a ten fold upregulation of cell surface expression (Miller et al., 1987; Sengelov et al., 1993). The principal ligands for Mac-1 are the coagulation factors fibrinogen (Altieri et al., 1990) and factor X (Altieri and Edgington, 1988), the complement C3 fragment iC3b (Ross and Lambris, 1982), and ICAM-1 (Diamond et al., 1991). However, Mac-1 is promiscuous and binds to denatured proteins (Davis, 1992). Neutrophil inhibitory factor (NIF), a hookworm glycoprotein, is an antagonist of Mac-1 (Moyle et al., 1994). Mac-1 is involved in the adhesion and transmigration of myeloid cells across endothelium, binding to and ingestion of iC3b-coated target cells, neutrophil homotypic aggregation, neutrophil and monocyte chemotaxis, binding and phagocytosis of opsonised particles (reviewed in Plow and Zhang, 1997).

1.1.1.c p150,95

p150,95 is expressed on myeloid cells, dendritic cells, activated B cells and some T cell populations. Its ligands are not well characterised. Like Mac-1, however, it is thought that p150,95 binds to iC3b (Bilsland et al., 1994), fibrinogen (Loike et al., 1991), denatured proteins (Davis, 1992), and perhaps ICAM-1 (Diamond et al., 1993). Recently, p150,95, but not LFA-1 or Mac-1, has been reported to mediate monocyte binding to collagen I (Garnotel et al., 2000).

1.1.1.d $\alpha_D\beta_2$

The most recently identified member of the leukocyte integrin family, $\alpha_D\beta_2$, is expressed on monocytes, macrophages, NK cells, a subpopulation of T and B cells, basophils, eosinophils and neutrophils. No binding of $\alpha_D\beta_2$ to ICAM-1 can be detected, but it does bind to ICAM-3 (Van der Vieren et al., 1995) and to vascular cell adhesion molecule (VCAM)-1 (Grayson et al., 1998).

1.1.2 Integrin structure and cation binding sites

Electron microscopy indicates that the amino-terminal (N-terminal) regions of the integrin α and β subunits form an extracellular globular head that is approximately 100 Å in diameter (Weisel et al., 1992). The more carboxy-terminal (C-terminal) portions of the α and β subunits form two long stalks, approximately 100 Å in length, which link the globular head to the membrane spanning helices. Each subunit has a short cytoplasmic tail.

1.1.2.a The alpha subunit

Within the extracellular region of the α subunit are seven N-terminal homologous repeats, each approximately 60 amino acids. An insertion of approximately 200 amino acids is present between repeats II and III in nine of the α subunits (α_1 , α_2 , α_{10} , α_{11} , α_L , α_M , α_X , α_D and α_E), and is known as the I (inserted/interactive) domain. The remainder of the α subunit, which is not characterised structurally, links to the transmembrane sequence and is followed by an approximately fifty residue cytoplasmic tail (Larson et al., 1989) (Fig. 1.2).

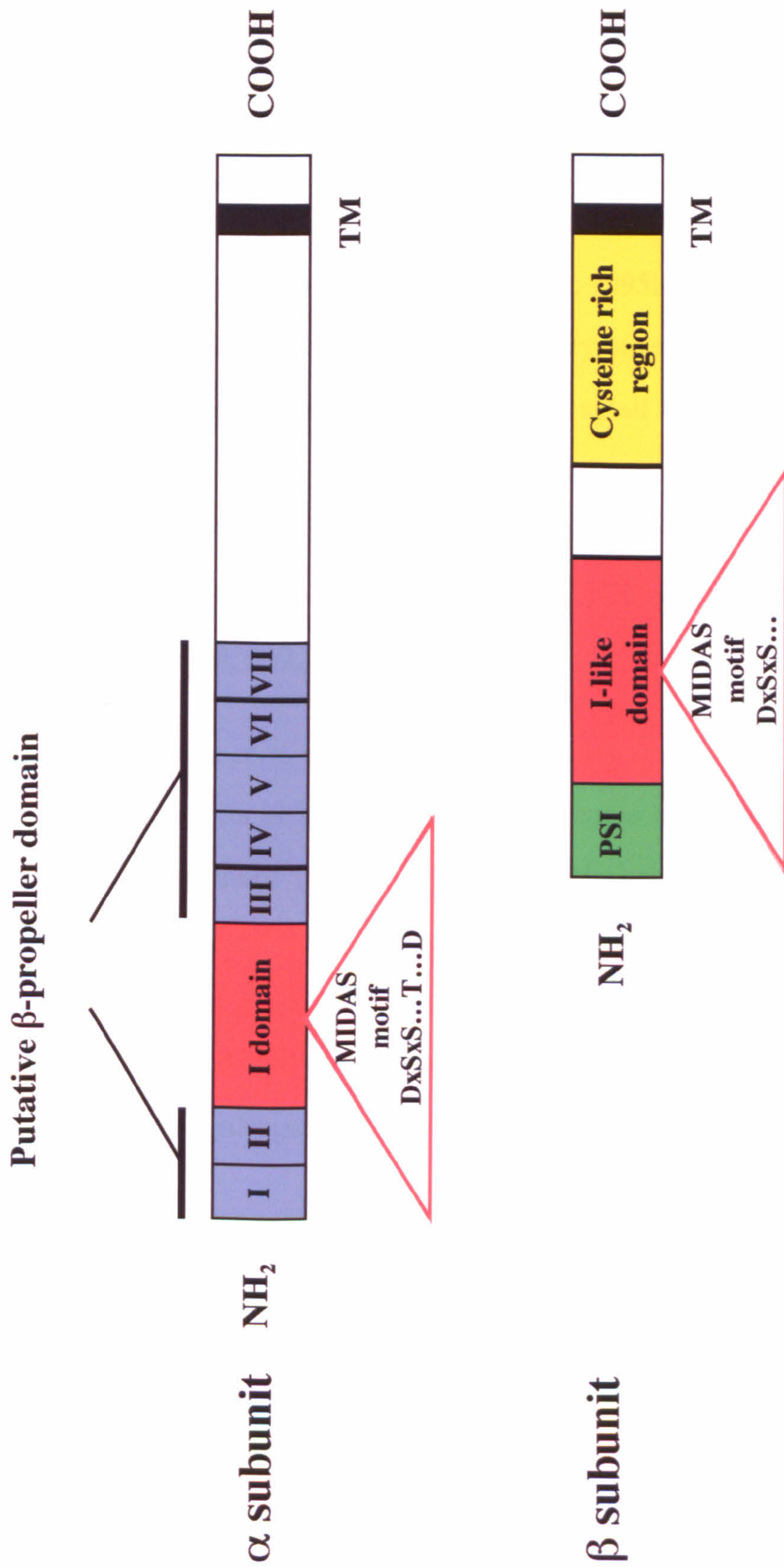


Figure 1.2 The domain organisation of an I domain containing integrin

The α subunit has seven repeat domains (blue) which are predicted to fold into a β -propeller domain. The I domain (red) is inserted between repeat II and III. The N-terminus of the β subunit has a region with homology to sequences in plexins and semaphorins (green). This PSI region is followed by a domain, conserved in all integrin β subunits, which is thought to have a similar structure to the I domain (red). At the C-terminus of the extracellular region is a domain in which 20% of the residues are cysteines (yellow). Both the I domain and conserved domain have a MIDAS motif. Each subunit has a short transmembrane region (TM) and an ~ 50 residue cytoplasmic tail.

(i) *The I domain*

The I domain folds autonomously (Huang and Springer, 1997) and, until recently, was the only integrin domain to be produced as an isolated recombinant protein. It is for this reason that the I domain is the only region for which detailed structural information is available. The structures of the I domains of α_L (Qu and Leahy, 1995), α_M (Lee et al., 1995a; Lee et al., 1995b), α_1 (Nolte et al., 1999) and α_2 (Emsley et al., 1997) have been solved. They have a classic dinucleotide binding fold, similar to that originally described for small G proteins, composed of a six stranded β -sheet, with five parallel strands and one anti-parallel strand, surrounded by seven α helices (Fig. 1.3). Helix 5 is shorter in LFA-1 due to a seven amino acid deletion (Qu and Leahy, 1995). A Mg^{2+}/Mn^{2+} binding site forms a crevice at the top of the domain (Lee et al., 1995b).

Michishita *et al.* were the first to identify a divalent cation binding site in an I domain (Michishita et al., 1993). It has become apparent that an Asp-X-Ser-X-Ser...Thr...Asp motif (where X is any amino acid), conserved in all integrin I domains, and termed the metal ion-dependent adhesion site (MIDAS), coordinates the cation (Lee et al., 1995b). For example, for LFA-1 the side chains of Ser¹³⁹, Ser¹⁴¹ and Asp²³⁹ directly coordinate the divalent cation while the Thr²⁰⁶ and Asp¹³⁷ side chains are hydrogen bonded to coordinating water molecules (Qu and Leahy, 1995).

(ii) *The N-terminal repeats*

The seven N-terminal repeats of the α subunit have been predicted to fold into a seven bladed β -propeller domain (Springer, 1997) (Fig. 1.4). This structure consists of seven β -sheets arranged cylindrically around a central cavity. Each β -sheet has four anti-parallel β -strands, with strand 1 closest to the centre and strand 4 on the outside of the structure. The angle of successive strands increases with



Figure 1.3 The crystal structure of the CD11a I domain

A ribbon diagram of the CD11a I domain crystal structure with bound Mn^{2+} (red), adapted from Qu and Leahy, 1995. The six β -strands (yellow) are surrounded by seven α -helices (blue).

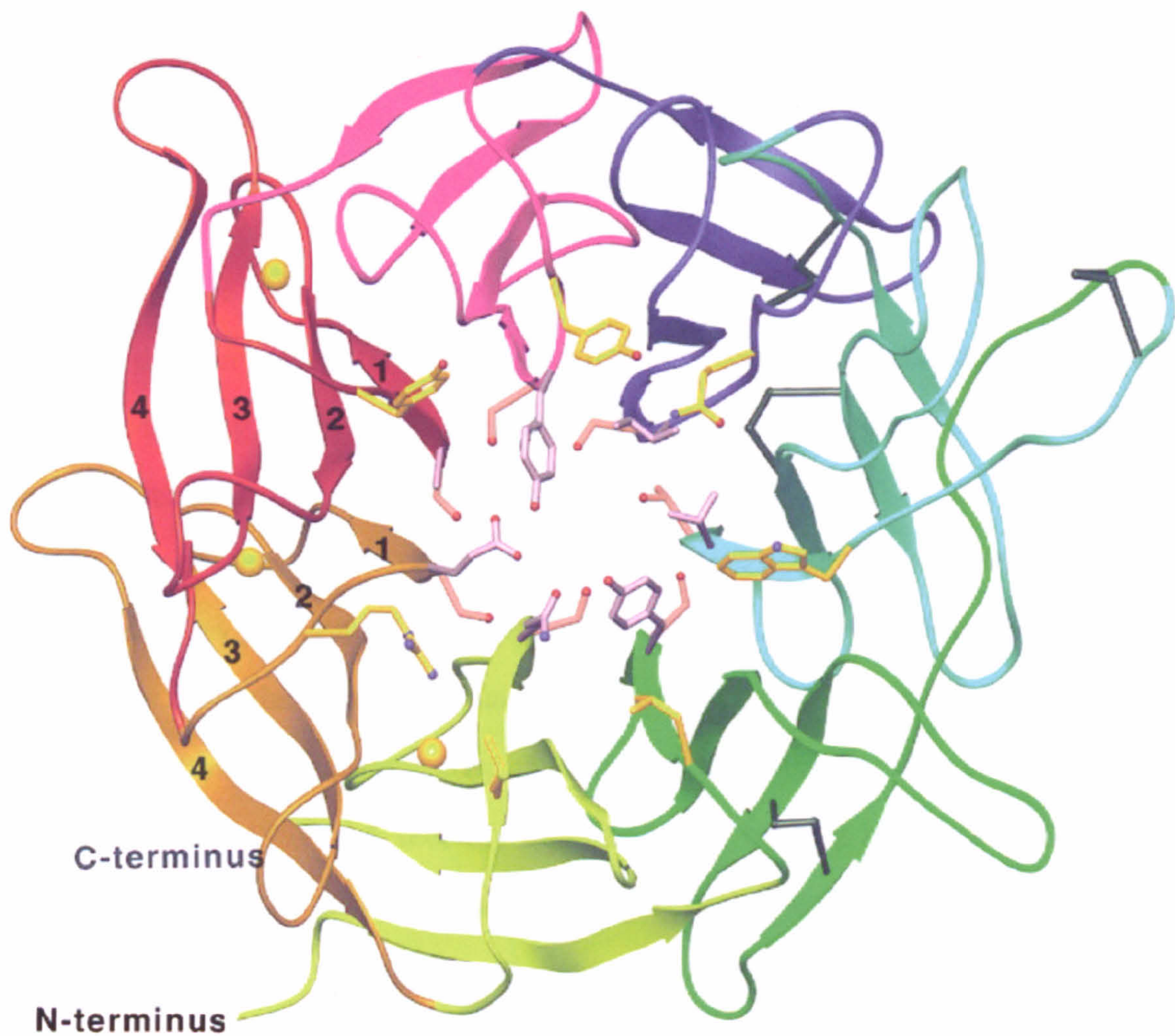


Figure 1.4 A model of an integrin β -propeller domain

A ribbon diagram of an integrin β -propeller domain viewed from the top, adapted from the model of Springer, 1997. Each of the seven blades of the propeller is shown in a different colour. The putative Ca^{2+} binding sites are indicated by yellow circles.

respect to the central axis, with the result that the overall structure resembles a propeller with each β -sheet representing one blade. The I domain is predicted to sit above the β -propeller domain, connected by a hydrophilic hinge (Springer, 1997). Folding of the β -propeller domain is reported to be dependent on association with the β subunit (Huang and Springer, 1997; Lu et al., 1998).

The three most C-terminal repeats (V-VII) or, in non-I domain containing integrins, the four most C-terminal repeats (IV-VII), each has a putative Ca^{2+} binding motif, resembling an EF hand sequence. The classical EF hand motif has a 13 residue consensus sequence with the side chains at positions 1, 3, 5, 7, 9 and 12 coordinating divalent cation. In the integrin sequence, however, a non-coordinating leucine occupies position 12. Another major difference between the integrin Ca^{2+} binding motif and the classical EF hands has been proposed by a model in which the integrin Ca^{2+} binding site is preceded and followed by β -strands rather than α -helices (Tuckwell et al., 1994). A recently described Ca^{2+} binding motif, present in the crystal structure of an alkaline protease, and termed a β -hairpin loop, fits with this type of model and also with the β -propeller model (Springer et al., 2000).

On the β -propeller model the putative Ca^{2+} binding sites lie close to each other on the lower surface of the domain (Springer, 1997). Interestingly, the mapping of a Ca^{2+} dependent monoclonal antibody (mAb) to discontinuous residues has provided support for the concept of the β -propeller model. Although these residues are almost 100 amino acids apart in the α subunit primary structure, on the β -propeller model they lie in adjacent loops on the lower surface of the domain and, notably, include a putative Ca^{2+} binding motif (Oxvig and Springer, 1998).

Although, as discussed above, there is experimental evidence to support the β -propeller model, circular dichroism (CD) studies on truncated α subunit proteins containing repeats IV-VII or III-VII indicate that the secondary structure consists of approximately 30% α -helix and 25% β -strand (Banères et al., 1998; Banères et al.,

2000). These data are not compatible with the β -propeller model, which contains only β -strands and coils. Further structural studies are therefore required.

(iii) *The C-terminal region*

Secondary structure predictions indicate that the extracellular C-terminal region of the α subunit folds into four to six β -sandwich domains (Lu et al., 1998).

1.1.2.b The beta subunit

The β subunit consists of four extracellular regions, the boundaries of which are ill defined. A cysteine-rich region at the N-terminus is followed by a region of approximately 240 amino acids that is highly conserved in the different β subunits as well as between species. This conserved region has homology with the α subunit I domain. The remainder of the extracellular portion of the β subunit forms another cysteine-rich region. The cytoplasmic tail is approximately 50 amino acids long (Kishimoto et al., 1987; Law et al., 1987; Tamkun et al., 1986) (Fig. 1.2).

(i) *The N-terminal cysteine rich region*

The N-terminal region of the β subunit has homology to the repeat regions in plexins and to a region in semaphorins, therefore it has been named the PSI domain (Plexins, Semaphorins and Integrins) (Bork et al., 1999). This region has seven cysteine residues, six of which disulphide bond to each other, but the first forms a long range disulphide bond to link to the C-terminal cysteine rich region. It is predicted that the PSI domain contains two α -helices, and is followed by four β strands that form a $\beta\beta$ sandwich (Huang et al., 2000).

(ii) *The β I-like domain*

It has been proposed that the conserved region folds in a similar manner to the α subunit I domain (Lee et al., 1995b; Tozer et al., 1996), and a feature of the conserved region is a putative divalent cation binding motif, Asp-X-Ser-X-Ser....Asp-X-Pro-Glu (where X is any amino acid), similar to the MIDAS motif. Recently, this region has been produced as an isolated protein which has 35% α -helix and 30% β -strand (Banères et al., 2000), compatible with an I domain-like structure. Structural algorithms, however, have provided conflicting predictions on whether or not the conserved region does adopt an I domain-like fold (Lin et al., 1997b; Tuckwell and Humphries, 1997). A model for the conserved region shows that, although it has some areas of uncertain topology, it is similar in structure to an I domain. The major difference between this model and an I domain are two long insertions, one between β -strands 2 and 3 and the other between β -strand 4 and α -helix 4. This model is supported by antibody mapping experiments (Huang et al., 2000). This region will therefore be referred to as the β I-like domain.

(iii) *The cysteine rich region*

C-terminal to the β I-like domain are four cysteine-rich repeats. Approximately 20% of the residues in these repeats are cysteines, indicating that the structure is likely to be rigid (Kishimoto et al., 1987; Law et al., 1987). It is predicted that this region forms approximately twenty β -strands that fold into $\beta\beta$ sandwiches (Huang et al., 2000).

1.1.2.c Association of integrin alpha and beta subunits

Mutations in integrin genes that lead to inherited diseases often result in failure of heterodimer formation. Most of these mutations occur in the β I-like domain. Recently, these mutations have been mapped on a model of the β I-like

domain and many cluster around the MIDAS motif (Hogg and Bates, 2000). Mutation of the MIDAS motif itself does not affect heterodimer formation however. These results suggest that the MIDAS face, but not the MIDAS itself, forms an interface with the α subunit.

It has been demonstrated that, although not adequate for cell surface expression, the N-terminal 301 amino acids of the β_2 subunit are sufficient for association with the α_L or α_M subunit (Tan et al., 2000). This provides further evidence that the β subunit interacts with the α subunit via the β I-like domain. There is increasing support for a model of the integrin quaternary structure in which the β subunit I-like domain contacts the β -propeller domain in the α subunit. More specifically, there is evidence that the top of the β I-like domain interacts with a loop between repeats 2 and 3 on the upper surface of the β -propeller domain, with the β subunit MIDAS motif orientated towards the loop. The opposite edge of the top of the β I-like domain is in close proximity with the bottom of the β -propeller in β -sheet 2, and the side of the domain in β -sheet 4 (Humphries et al., 2000; Puzon-McLaughlin et al., 2000; Zang et al., 2000). The α subunit stalk region may also be involved in heterodimer formation, as mutation of this region disrupts interaction with the β subunit (Lu et al., 1998; Zeller et al., 1998). A model of the integrin quaternary structure is shown in **Figure 1.5**.

1.1.3 Ligand binding sites in integrins

A number of sites in both the α and β subunits of integrins have been identified as ligand contact points. The three major regions are the I domain and the N-terminal repeats in the α subunit and the I-like domain of the β subunit. It is likely that all these sites must cooperate for binding to ligand to be of sufficient affinity to support cell adhesion. However, whether they combine to form the complete ligand binding face or operate in a sequential manner has yet to be unequivocally established.

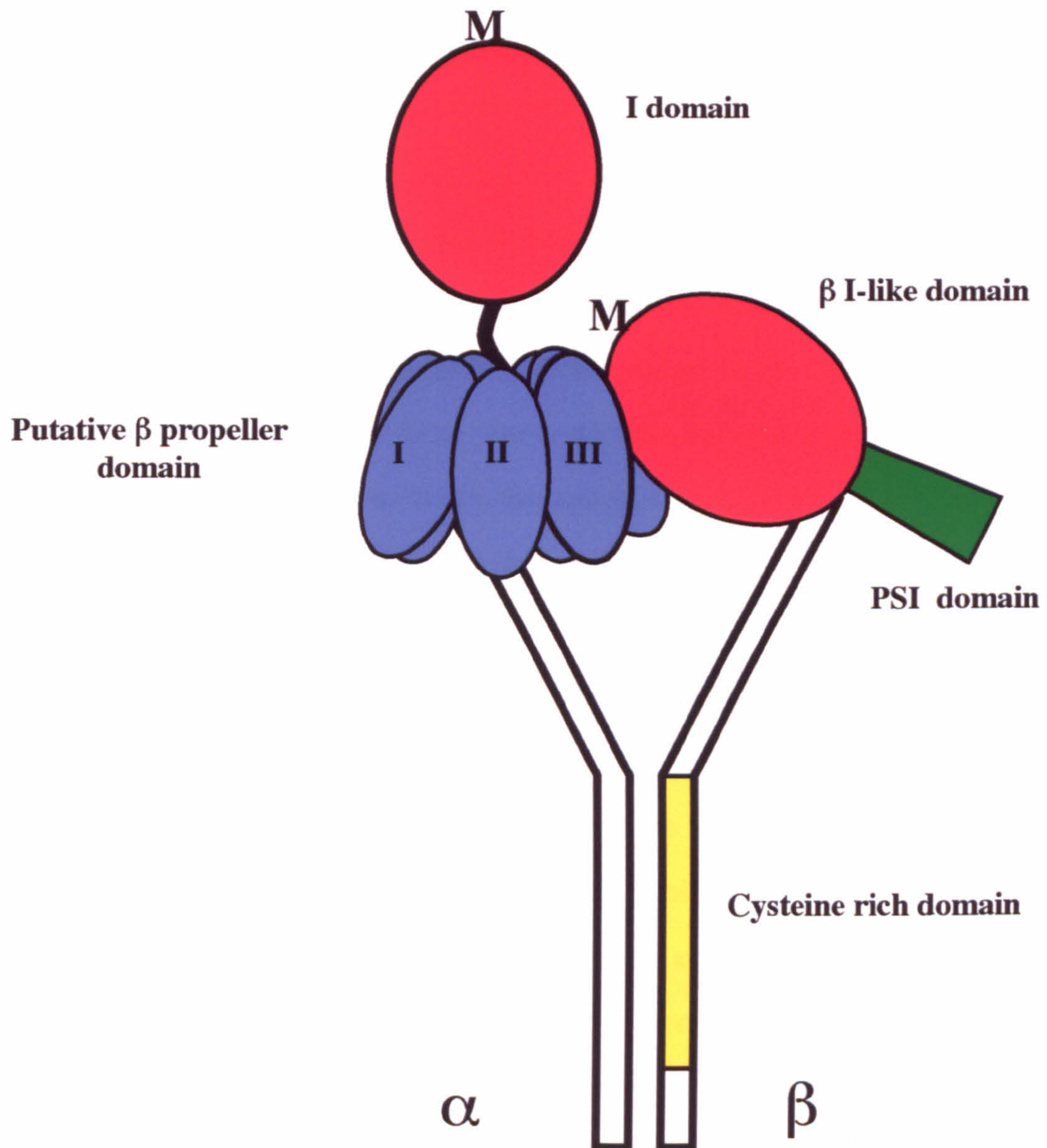


Figure 1.5 A hypothetical model of the quaternary structure of an integrin
The model shows the I domain sitting above the β -propeller domain in the α subunit. The I-like domain of the β subunit contacts the β -propeller between repeats II and III. The MIDAS face on the I domain and β I-like domain are indicated by 'M'.

1.1.3.a The alpha subunit

(i) *The I domain*

I domains, when present, play an important role in ligand binding. The first inference that the I domain is important in regulating integrin function came when cloning of integrin α subunits revealed homology to the A domains of von Willebrand Factor, which had been shown to interact with ligand (Larson et al., 1989). Further evidence came when the epitopes of both function blocking and activating mAbs were mapped to this domain (Champe et al., 1995; Landis et al., 1993; Randi and Hogg, 1994). Binding of recombinant I domains to ligands demonstrated a direct role in ligand binding. For example, the I domain from LFA-1 has been shown to bind to ICAM-1 (Randi and Hogg, 1994), ICAM-2 (Edwards et al., 1998) and ICAM-3 (van Kooyk et al., 1996); the Mac-1 I domain binds to iC3b (Ueda et al., 1994), fibrinogen, ICAM-1 (Zhou et al., 1994), ICAM-2 (Xie et al., 1995) and neutrophil inhibitory factor (Muchowski et al., 1994; Rieu et al., 1994); the $\alpha_D\beta_2$ I domain binds to VCAM-1 (Van der Vieren et al., 1999); the α_1 I domain binds collagen I, collagen IV and laminin (Calderwood et al., 1997); the α_2 I domain binds collagens I, II, IV and XI (Kamata and Takada, 1994; Tuckwell et al., 1995; Tuckwell et al., 1996) and echovirus 1 (King et al., 1997).

Mutation of any of the I domain MIDAS motif residues abolishes ligand binding (Edwards et al., 1995; Kamata et al., 1995; McGuire and Bajt, 1995; Michishita et al., 1993), demonstrating the critical role of the divalent cation in ligand binding. It has been suggested that the divalent cation bound to the I domain may directly participate in ligand binding by interacting with an acidic residue in the ligand. The crystal lattice of the α_M I domain, in which the bound Mg^{2+} is coordinated by a glutamic acid side chain in an adjacent molecule, supports this idea (Lee et al., 1995b). All the known integrin ligands contain an acidic residue in their

binding motif, for example Glu³⁴ in ICAM-1. Mutation of this residue in ICAM-1 abolishes adhesion to the LFA-1 I domain (Stanley and Hogg, 1998). Another example is the triplet Gly-Glu-Arg in a peptide from collagen I, which is essential for binding of this peptide to the α_2 I domain (Knight et al., 1998). The recent co-crystallisation of the α_2 I domain complexed with a collagen peptide directly demonstrates that the Glu of the Gly-Glu-Arg motif coordinates Mg²⁺ bound to the I domain MIDAS motif (Emsley et al., 2000). However, this structure contradicts the evidence that ligand binding to the α_2 I domain results in displacement of the divalent cation to form a divalent cation free ligand-I domain complex (Dickeson et al., 1997).

Other residues that are essential for α_L , α_M and α_2 I domain binding to ligand are located on the surface of the I domain surrounding the MIDAS motif, indicating that this face contacts ligand (Edwards et al., 1998; Huang and Springer, 1995; K  pyl   et al., 2000; Rieu et al., 1996; Smith et al., 2000a; Zhang and Plow, 1997). These results, obtained by mutagenesis, were confirmed by the co-crystallisation of the α_2 I domain complexed with a collagen peptide (Emsley et al., 2000). The critical residues are not well conserved, therefore may provide specificity for ligand binding. In support of this, mutational analysis has identified additional residues on the MIDAS face of the α_L I domain required for ICAM-2 binding in comparison to ICAM-1 binding (Edwards et al., 1998). For the collagen binding I domains, however, these residues are invariant suggesting that these I domains bind collagen in a similar manner (Emsley et al., 2000).

(ii) *The N-terminal repeats of I domain containing integrins*

A chimeric form of LFA-1, in which the murine I domain replaces the human I domain, does not bind human ICAM-1 (Edwards et al., 1995), and LFA-1 from which the I domain has been removed does not bind ICAM-1 (Leitinger and Hogg, 2000; Yalamanchili et al., 2000). These results demonstrate that the I domain is

essential for LFA-1 binding. Intact LFA-1 mediates stable adhesion to lipid bilayers containing ICAM-1 under flow conditions. In the same system, however, a GPI linked LFA-1 I domain supports rolling adhesion (Knorr and Dustin, 1997). Other sites in the intact integrin must therefore cooperate with the I domain to allow stable adhesion to ligand. Another region of the N-terminal of the LFA-1 α chain, repeated domains V and VI, has been shown to bind ICAM-1 in an *in vitro* translation system (Stanley et al., 1994). In this study it was observed that binding to ICAM-1 was much stronger when the protein was extended to include the I domain. A role for these repeated domains in ligand binding has also been demonstrated for α_2 . Recombinant α_2 I domain binding to collagen is enhanced when the recombinant protein is extended to include the first putative Ca^{2+} -binding motif. This effect is prevented by mutation of the Ca^{2+} -binding motif (Dickeson et al., 1997). For Mac-1, iC3b, which binds to the isolated α_M I domain (Ueda et al., 1994), also binds to Mac-1 from which the I domain has been deleted (Yalamanchili et al., 2000). These results indicate that, although the I domain is a major ligand binding site in those integrins that contain them, sequences outside the I domain also contribute to binding.

(iii) *The N-terminal repeats of integrins without I domains*

In integrins that do not have an I domain, the N-terminal repeat domains appear to be the main ligand binding site in the α subunit. Many of the mutations of the repeated domains that diminish ligand binding lie in loops on the upper surface of the β -propeller model. For example, for α_5 residues in the 2-3 loop of repeat III (Irie et al., 1995), loops in repeat II and IV of α_4 (Irie et al., 1997), the 4-1 loop between repeats II and III of α_5 (Mould et al., 1998), the boundary of repeat I and II and the 4-1 loop at the boundary of repeats II and III of α_3 (Zhang et al., 1999), residues 184-193 in the 2-3 loop in repeat III and residue 224 of the 4-1 loop between repeats II

and III of α_{Ib} (Kamata et al., 1996; Tozer et al., 1999) have all been shown to be important for interaction with ligand.

A number of early studies indicated that the putative cation binding sites in the N-terminal repeats directly interact with ligand (D'Souza et al., 1990; D'Souza et al., 1991; Masumoto and Hemler, 1993). These sites lie on the bottom of the β -propeller model, on the opposite side of the domain to the predicted ligand binding surface. Therefore, if the β -propeller model is correct, it is difficult to conceive how ligand can bind to both sides of the domain. Integrins in which the putative Ca^{2+} binding sites in the repeat domains are mutated are often not expressed on the cell surface (Basani et al., 1996; Gidwitz et al., 2000). Conservative mutations, however, allow expression and indicate that these sites may not directly bind ligand but may be involved in adhesion strengthening (Pujades et al., 1997).

It is of interest that VCAM-1, which binds to the non-I domain integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$, also binds to the I domain of $\alpha_D\beta_2$ (Van der Vieren et al., 1999). The Asp⁴⁰ residue of VCAM-1, which is crucial for binding to $\alpha_4\beta_1$ and $\alpha_4\beta_7$, is also essential for binding to the $\alpha_D\beta_2$ I domain, suggesting a common mechanism for ligand binding in I domain containing and non-I domain containing integrins.

1.1.3.b The beta subunit

Other than the I domain, the only region of an integrin to be shown to directly bind ligand is the β I-like domain. A recombinant β_3 I-like domain binds to fibrinogen and to an RGD containing peptide (Cierniewski et al., 1999). A peptide corresponding to the C-terminus of the fibrinogen γ chain does not bind to the I-like domain, but to a distinct site elsewhere in $\alpha_{\text{Ib}}\beta_3$ (Cierniewski et al., 1999).

Mutation of the MIDAS-like motif residues Asp¹³⁴, Ser¹³⁶, Asp²³² and Glu²³⁵ in β_2 prevents LFA-1 and Mac-1 binding to their ligands (Bajt et al., 1995; Goodman

and Bajt, 1996). Homologous residues in other β subunits are also critical for ligand binding (Bajt and Loftus, 1994; Lin et al., 1997b; Puzon-McLaughlin and Takada, 1996; Tozer et al., 1996). Peptides covering the β subunit Asp-X-Ser-X-Ser motif inhibit ligand binding and have been shown to directly bind to ligands and RGD containing peptides (D'Souza et al., 1994; Liu et al., 1997).

$\alpha_4\beta_1$ does not bind to mucosal addressin cell adhesion molecule (MAdCAM)-1, whereas $\alpha_4\beta_7$ does. The region of the β subunit involved in determining this specificity of ligand binding is the putative I domain, as swapping this region of β_1 for that of β_7 enables the chimeric $\alpha_4\beta_{1/7}$ to bind to MAdCAM-1 (Tidswell et al., 1997). This is also the case for other β subunits: when a highly divergent sequence in β_1 is replaced with the corresponding residues of β_3 the mutated $\alpha_v\beta_1$ integrin recognises $\alpha_v\beta_3$ ligands (Takagi et al., 1997), and a similar region of β_3 substituted into β_5 allows binding to fibrinogen (Lin et al., 1997a).

Although a recombinant α_M I domain has been shown to bind iC3b (Ueda et al., 1994), cells transfected with the α_M or the β_2 subunit individually do not bind to iC3b. However, cells transfected with α_M and a truncated form of the β_2 subunit (residues 23-457), containing the β I-like domain, bind to iC3b in a manner similar to the complete $\alpha_M\beta_2$ heterodimer (Goodman et al., 1998). These results imply that the affinity of the interaction of the individual subunits is not high enough to support cell binding, and that the I-like domain of the β_2 subunit cooperates with the α_M subunit for high affinity binding.

1.1.4 Conformational changes in integrins

A change in the shape of integrins upon activation has been demonstrated by fluorescence resonance energy transfer (Sims et al., 1991) and electron microscopy (Du et al., 1993), but details of the structural changes that occur are not yet known. Three pieces of evidence indicate that one of the changes that occurs is movement of

the α subunit relative to the β subunit. A mAb epitope, which is located in the stalk region of the β_1 subunit, is masked when complexed with the α_5 subunit but revealed upon ligand binding (Tsuchida et al., 1998). A second mAb, which recognises an epitope spanning the α_L and β_2 subunits, preferentially recognises activated LFA-1 (Zang et al., 2000). Finally, electron microscopy shows a separation of the α and β chain globular heads upon binding ligand mimetic peptides (Hantgan et al., 1999).

The Mac-1 activation dependent epitope recognised by mAb CBRM1/5 has been mapped to the I domain, close to the MIDAS motif, suggesting that a conformational change in the I domain also occurs (Oxvig et al., 1999). For some time there has been evidence from crystallographic studies for different conformations of the I domain. Lee *et al.* showed that the structure of the α_M I domain crystallised in the presence of Mg^{2+} differed from that crystallised in the presence of Mn^{2+} (Lee et al., 1995a; Lee et al., 1995b). In the former structure a Glu side chain from a neighbouring I domain in the crystal contributed to the coordination of the Mg^{2+} ion. It was proposed that the Glu would normally be donated by ligand and that these structures therefore represented the active (ligand-bound) and inactive (unliganded) conformation respectively. Others, however, did not find any major differences in structure (Baldwin et al., 1998; Qu and Leahy, 1995; Qu and Leahy, 1996), consequently there has been some debate on whether or not there is an “active” conformation of the I domain, or whether it is a crystal lattice artefact (Baldwin et al., 1998; Liddington and Bankston, 1998). The recently solved structure of the α_2 I domain complexed with a peptide from collagen resolves this issue by demonstrating that a ligand-induced conformational change does occur (Emsley et al., 2000). Moreover, the differences between the two forms of the α_M I domain are nearly identical to the changes seen between unliganded and collagen-bound α_2 I domain. The alterations which occur upon the shift from the inactive to the active conformation include loss of direct coordination of the metal ion by an Asp and the formation of a direct bond to a Thr and Glu from a neighbouring

molecule (or ligand) providing the sixth metal coordination site. These modifications in cation coordination result in significant changes in the conformation of the MIDAS face and propagate through to the opposite face of the domain, altering the position of the C-terminal α -helix (Emsley et al., 2000; Huth et al., 2000; Li et al., 1998).

Evidence for the functional importance of changes in I domain structure has been obtained by mutagenesis. Substitution of residues in the hydrophobic core of the I domain can activate ligand binding (Huth et al., 2000; Li et al., 1998; Shimaoka et al., 2000; Xiong et al., 2000). Mutations on the bottom of the I domain (i.e. on the opposite side of the molecule to the MIDAS face) also modulate activation (Oxvig et al., 1999; Zhang and Plow, 1996). The fungal metabolite lovastatin binds to a hydrophobic pocket on the bottom of the LFA-1 I domain and locks it in its inactive conformation. Lovastatin thus acts as a small molecule antagonist of LFA-1, inhibiting binding to ICAM-1 without binding to the MIDAS face (Kallen et al., 1999). These results suggest the bottom of the I domain is involved in allosteric control of ligand binding to the MIDAS face.

There are several lines of evidence which support the idea that the cysteine rich region in the β subunit is involved in regulating adhesion. Firstly, the epitopes of a number of activation dependent mAbs have been located in this region (Du et al., 1993; Huang et al., 2000; Stephens et al., 1995). Secondly, chimeric LFA-1 in which the β_2 subunit cysteine-rich region is replaced with that of the β_1 subunit is constitutively active for binding ICAM-1 (Douglass et al., 1998). Finally, a single amino acid substitution in the cysteine rich region of β_3 renders $\alpha_{\text{Ib}}\beta_3$ constitutively active for ligand binding (Kashiwagi et al., 1999). These results suggest that the cysteine rich region must be involved in a change in integrin conformation upon activation.

1.1.5 LFA-1 activation

Integrin activation is tightly regulated by a complex interplay between cation binding, signalling events, associations with the cytoskeleton and interaction with other accessory molecules. On resting cells LFA-1 is in an inactive form which is unable to mediate stable binding to the ICAMs. Ligation of cell surface receptors, such as the T cell receptor (Dustin and Springer, 1989), the costimulatory molecule CD28 (Shimizu et al., 1992) or chemokine receptors (Campbell et al., 1998) generates intracellular signals that lead to activation of LFA-1-mediated cell adhesion. This is termed “inside-out” activation. Artificial agents, such as phorbol esters, which are activators of protein kinase C (PKC), can be used as a means to induce intracellular signalling leading to LFA-1 activation. LFA-1 can also be activated by “outside-in” mechanisms, for instance by the addition of Mn^{2+} or Mg^{2+} in the presence of EGTA to chelate Ca^{2+} (Dransfield et al., 1992; Stewart et al., 1996). A number of mAbs directed against either the α or β subunit of LFA-1 can directly cause activation (Landis et al., 1993; Stephens et al., 1995). Both the divalent cations and the activating mAbs bind to the extracellular portion of LFA-1, thereby inducing a conformational change that reveals or forms a ligand contact site and unmasks the epitope recognised by mAb 24. There is evidence that activation from the outside increases the affinity of LFA-1 for its ligands, but that inside-out activation does not (Stewart et al., 1996). Therefore, it has been proposed that inside-out activation involves an increase in the avidity of LFA-1 interaction (Stewart et al., 1996). The factors reported to contribute to LFA-1 activation are discussed below and are summarised in **Figure 1.6**. The mechanisms of LFA-1 activation constitute the subject matter of this thesis, and will be discussed in detail in the following chapters.

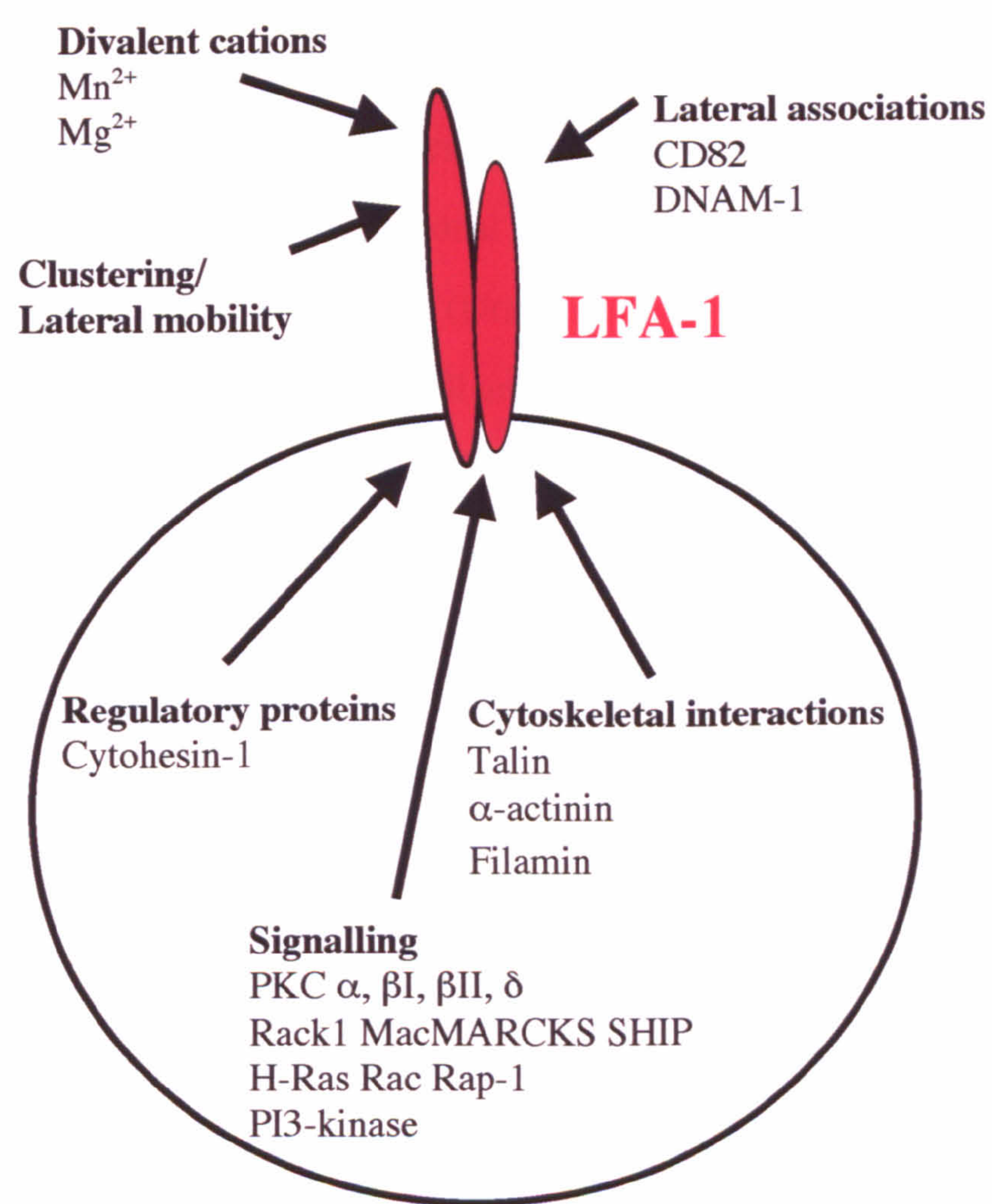


Figure 1.6 Summary of the factors that are reported to contribute to LFA-1 activation

1.1.5.a Divalent cations

Integrin binding to ligand requires physiological concentrations of divalent cations. Mg^{2+} and Mn^{2+} induce activation of LFA-1 and adhesion to ligand, presumably by inducing a conformational change, (Dransfield et al., 1992). In contrast, Ca^{2+} inhibits LFA-1 adhesion (Dransfield et al., 1992). These effects of divalent cations on integrin-mediated cell adhesion may be a consequence of cation binding to the I domain MIDAS motif. Evidence for this comes from the fact that the cation binding properties of recombinant I domain, and the cation-dependency of ligand binding to the recombinant I domain or intact integrin, are similar (Michishita et al., 1993; Ueda et al., 1994). Mutations in the I domain which abolish cation binding also abolish ligand binding (McGuire and Bajt, 1995; Michishita et al., 1993).

Removal of Ca^{2+} activates integrin adhesion (Dransfield et al., 1992), and also weakens the association of integrin α and β subunits (Dustin et al., 1992). Putative Ca^{2+} binding motifs are predicted to lie close to one another on the bottom of the β -propeller domain (Springer, 1997) (see **Fig. 1.4**), therefore Ca^{2+} may regulate the association of this region with the β subunit and thereby regulate ligand binding. As discussed in **Chapter 1.1.2.c**, there is evidence that the β -propeller does interact with the β subunit I-like domain.

1.1.5.b The LFA-1 cytoplasmic tail and cytoskeletal interactions

There is growing evidence that in its non-active state LFA-1 is tethered to the cytoskeleton and upon cell activation these cytoskeletal associations are altered, allowing LFA-1 to move in the membrane (Kucik et al., 1996; Zhou and Li, 2000). It has been demonstrated that deletion of the entire α subunit or β subunit cytoplasmic tail results in constitutively active LFA-1 (Tan et al., 2000; van Kooyk

et al., 1999). The lack of the cytoplasmic tails means that mobility of the integrin cannot be constrained by the cytoskeleton and so these forms of the integrin cluster, facilitating adhesion. Mutation of the membrane proximal residues, KVGFFKR in the α subunit and DLRE in the β subunit, also result in constitutively active LFA-1 (van Kooyk et al., 1999). It is thought that these residues form a salt bridge between the subunits that keeps the integrin in its inactive state (Hughes et al., 1996). Mutation of amino acids more C-terminal in the β_2 tail results in LFA-1 that cannot bind ICAM-1 (Hibbs et al., 1991).

Despite much research on the subject it is still unclear how LFA-1 interacts with the cytoskeleton and how these associations are altered upon cell activation. The cytoskeletal proteins talin (Sampath et al., 1998), α -actinin (Sharma et al., 1995) and filamin (Sharma et al., 1995) all bind to a peptide corresponding to the β_2 tail. However, convincing evidence that these associations occur directly *in vivo* is lacking.

1.1.5.c Signalling

The signalling events involved in the activation of LFA-1 are still poorly understood, although there is a growing list of second messengers that are reported to alter LFA-1 activity. There is recent evidence that upon activation LFA-1 associates with specialised membrane regions known as lipid rafts, which act as platforms for signalling molecules and adapter proteins (Krauss and Altevog, 1999).

It has been known for many years that activation of protein kinase C (PKC) with phorbol esters triggers LFA-1 mediated adhesion. More recently it has been demonstrated that the conventional PKC isoforms, α , β I and β II and the novel PKC, PKC δ , but not the atypical PKC ζ , activate LFA-1 (Katagiri et al., 2000). The receptor for activated protein kinase C (Rack1) associates with the integrin β_2 subunit upon phorbol ester stimulation of the lymphoblastoid cell line, JY (Liliental and

Chang, 1998). It is thought that this interaction serves to recruit activated PKC to sites of cell adhesion, and may thereby regulate LFA-1-cytoskeleton interaction. Macrophage-enriched myristoylated alanine-rich C kinase substrate (MacMARCKS) is a PKC substrate that is involved in regulation of LFA-1 adhesion in macrophages. Phosphorylation of MacMARCKS by PKC results in LFA-1 diffusion in the membrane and induces LFA-1-mediated adhesion (Zhou and Li, 2000). It is speculated that MacMARCKS is involved somehow in maintaining a cytoskeletal complex that restrains LFA-1 movement and that this restraint is released upon MacMARCKS phosphorylation. Another protein downstream of PKC is SH2-containing inositol phosphatase (SHIP), overexpression of which enhances phorbol ester-induced LFA-1 activation by an as yet unidentified pathway (Rey-Ladino et al., 1999)

Cytohesin-1 is a guanine nucleotide exchange factor (GEF) for ADP-ribosylation factor (ARF) GTPases that is expressed predominantly in haematopoietic cells (Meacci et al., 1997). It has a central SEC7 domain that directly interacts with the integrin β_2 subunit, and a C-terminal PH domain that binds to phosphatidylinositol-(3,4,5)-trisphosphate (PIP₃) (Kolanus et al., 1996; Nagel et al., 1998). Activation of phosphatidylinositol-3-OH kinase (PI3-kinase) generates PIP₃, resulting in recruitment of cytohesin-1 to the membrane, where it interacts with the integrin β_2 subunit cytoplasmic tail. This interaction induces cell spreading through the ARF-GEF activity of cytohesin-1, thus activating LFA-1-mediated adhesion (Geiger et al., 2000; Nagel et al., 1998). The small GTPase H-Ras has been shown to lie downstream of chemokine receptors and, similarly to Rac, activates LFA-1 adhesion through PI3-kinase (Katagiri et al., 2000; Tanaka et al., 1999). Another small GTPase, Rap-1, is activated following ligation of the T cell receptor (TCR) or CD31 and also lies in the pathway to LFA-1 activation (Katagiri et al., 2000; Reedquist et al., 2000).

1.1.5.d Lateral associations

Lateral associations of integrins with other membrane proteins also modulate integrin activity. Molecules that have been shown to form lateral associations with integrins are either tetraspans or members of the immunoglobulin superfamily (IgSF) (Hemler, 1998). The tetraspans are thought to interact with the integrin α subunits and to recruit signalling proteins to the complex. They have been implicated in the control of cell migration. The only tetraspan shown to be associated with LFA-1 is CD82 (Shibagaki et al., 1999). T cell stimulation upregulates CD82 expression leading to association with LFA-1 and stimulation of LFA-1 mediated adhesion. DNAM-1 (CD226) is a member of the IgSF that associates with LFA-1 in stimulated T cells (Shibuya et al., 1999). This association requires the PKC-mediated phosphorylation of Ser³²⁹ in DNAM-1. Engagement of LFA-1 then augments tyrosine phosphorylation of the protein tyrosine kinase Fyn. Fyn is thought to be responsible for phosphorylation of Tyr³²² of DNAM-1 and this enables DNAM-1 to transduce signals which initiate NK or T cell cytotoxic responses against target cells.

1.2 THE INTERCELLULAR ADHESION MOLECULES

To date five Intercellular Adhesion Molecules (ICAMs) have been identified, namely ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), ICAM-4 (LW, CD242), and ICAM-5 (telencephalin) (Fig. 1.7). Each of these proteins has a distinct cell and tissue distribution. The ICAMs are members of the immunoglobulin supergene family (IgSF) and are type I integral membrane glycoproteins composed of immunoglobulin (Ig)-like extracellular domains, a single spanning transmembrane region, and a short C-terminal cytoplasmic tail (Simmons et al., 1988; Staunton et al., 1988). The N-terminal domain of all five ICAMs contains a ligand binding site for the leukocyte integrin LFA-1. A Glu residue, conserved in all but ICAM-4, is critical for this interaction. As the cytoplasmic tails of the ICAMs have low identity they may have different associations and deliver distinct signals within the cell.

1.2.1 ICAM-1

ICAM-1 was the first ligand to be described for LFA-1 (Marlin and Springer, 1987; Rothlein et al., 1986). It is a 55 kDa polypeptide with five Ig-like extracellular domains. Differential glycosylation of the eight potential N-linked glycosylation sites produces heterogeneity in molecular weight of 80-115 kDa, depending on cell origin. In non-inflammatory situations ICAM-1 is constitutively expressed at low levels on various cells of haematopoietic and non-haematopoietic origin, including leukocytes, endothelium, epithelium and fibroblasts. Inflammatory mediators, such as the cytokines interleukin (IL)-1 β , tumour necrosis factor (TNF)- α , and interferon (IFN)- γ , initiate a transcription-dependent upregulation of ICAM-1 cell surface expression. The gene encoding ICAM-1 is located on chromosome 19 band p13.3-13.4 (Trask et al., 1993) and has a complex promoter containing consensus sequences for multiple transcription factors, including SP-1, AP-1, AP-2, AP-3, Ets-1 and NF- κ B. The signal transduction pathways and transcription complexes that

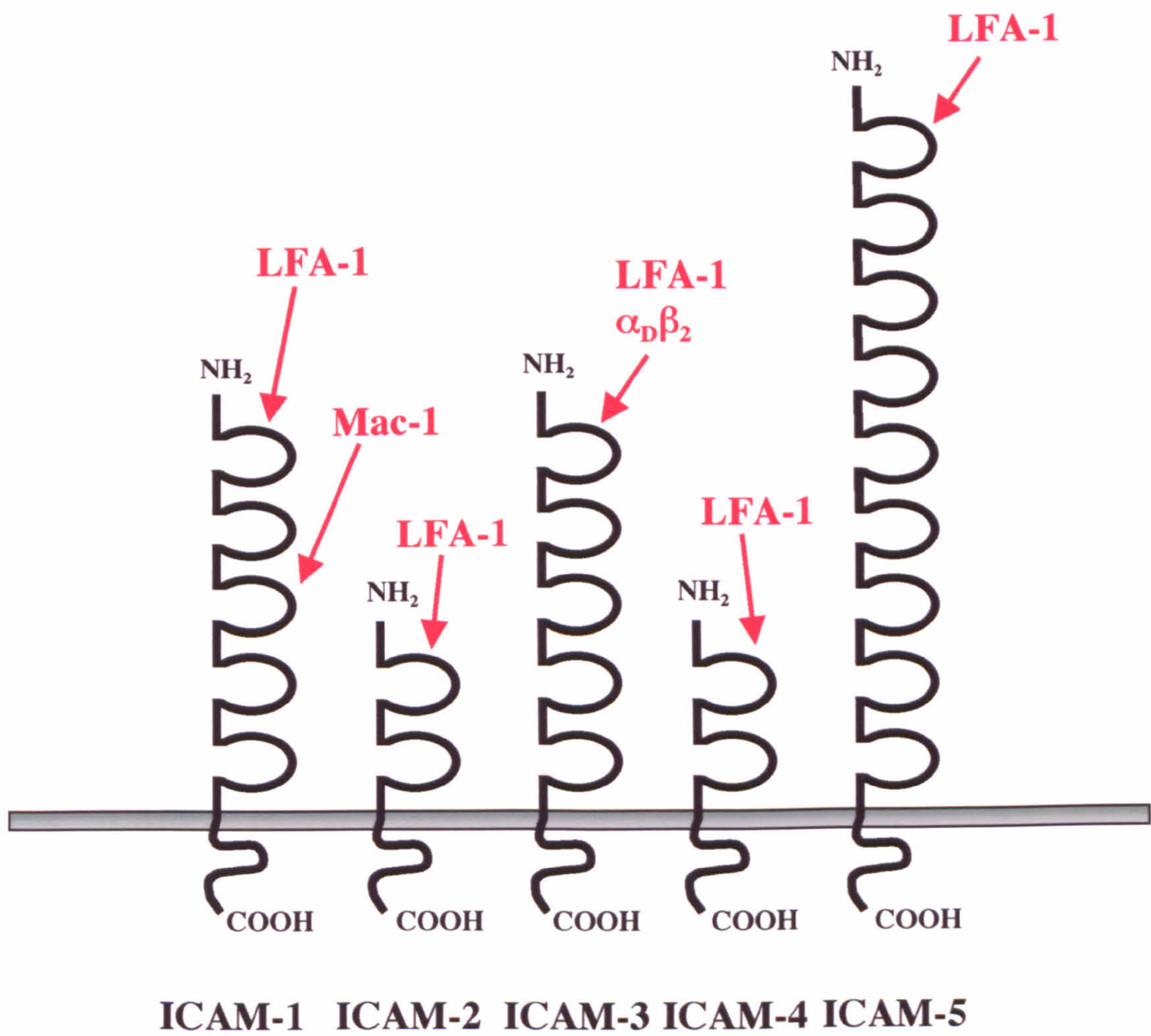


Figure 1.7 The ICAM family of adhesion molecules
A schematic diagram of the domain structure of the ICAMs. The domains to which their ligands bind are indicated.

regulate ICAM-1 expression are complex and display cell type and stimulus specificity (reviewed in (Roebuck and Finnegan, 1999)). ICAM-1 can also transmit signals into the cell (see **Chapter 1.3.2.d**).

A form of ICAM-1 (sICAM-1), thought to be composed of the five extracellular domains of the molecule, is secreted by activated leukocytes and endothelium. It is found in the serum of healthy individuals at a concentration of 100-200ng/ml (Rothlein et al., 1991; Seth et al., 1991) and has been reported to be elevated in inflammation, infection and cancer. No alternative splice forms of ICAM-1 mRNA have been observed, therefore sICAM-1 is thought to be the result of protease-mediated shedding. sICAM-1 retains the ability to bind LFA-1, and it has been speculated that by acting as a competitor it may inhibit cell-cell adhesion and "switch off" the immune response (reviewed in (Gearing and Newman, 1993)).

1.2.2 ICAM-2

ICAM-2 (CD102) is encoded by a gene located on chromosome 17 q23-q25 (Sansom et al., 1991). It has a 28 kDa polypeptide backbone, but glycosylation of the six N-linked sites produces a 55-65 kDa protein. The extracellular portion of ICAM-2 forms two Ig-like domains, which are most homologous to the two N-terminal domains of ICAM-1, showing 34% amino acid identity (Staunton et al., 1989a). ICAM-2 is constitutively expressed by lymphocytes and monocytes, but not neutrophils, as well as vascular endothelium (de Fougerolles et al., 1991) and platelets (Diacovo et al., 1994). In contrast to ICAM-1, expression is down-regulated at the transcriptional level on endothelium by the inflammatory cytokines IL-1 β and TNF- α (McLaughlin et al., 1998).

It had been postulated that ICAM-2 had a role in lymphocyte recirculation, but data from ICAM-2 knockout mice does not support this. Rather, these mice exhibit elevated and prolonged eosinophil accumulation in allergic lung interstitium,

with a resultant airway hyperresponsiveness (Gerwin et al., 1999). Consistent with the fact that megakaryocytes express ICAM-2 but not ICAM-1 or ICAM-3 (Diacovo et al., 1994), the ICAM-2 deficient mice have a reduced number of these cells in the bone marrow (Gerwin et al., 1999). There is evidence that on NK target cells ICAM-2 and the cytoskeletal protein ezrin become concentrated in the uropod, and thus the target cells become sensitive to NK activity (Helander et al., 1996). ICAM-2 is thought to bind directly to the ERM (Ezrin/Radixin/Moesin) family of proteins via a positively charged amino acid cluster in the membrane proximal region of the cytoplasmic tail (Yonemura et al., 1998).

1.2.3 ICAM-3

ICAM-3 was cloned in 1992/93 by three groups (de Fougères et al., 1993; Fawcett et al., 1992; Vazeux et al., 1992). It has a core polypeptide of 57 kDa comprising a 37 amino acid cytoplasmic tail, 25 amino acid transmembrane region and 456 amino acid extracellular domain. Cell surface ICAM-3 has a molecular mass between 116 and 140 kDa, depending on the degree of glycosylation of the 15 potential N-linked glycosylation sites. Like ICAM-1, ICAM-3 has five extracellular Ig-like domains and is encoded by a gene located on chromosome 19 p13.3-13.2 (Bossy et al., 1994). The extracellular domains of ICAM-3 share 48% amino acid identity with ICAM-1 and 31% with ICAM-2, however there is little conservation of the cytoplasmic and transmembrane domains. ICAM-3 has a 37 amino acid cytoplasmic tail with five serine and two tyrosine residues, whereas ICAM-1 and ICAM-2 do not contain serines and have only one tyrosine. ICAM-3 is constitutively expressed at high levels on all leukocytes but, unlike ICAM-1 and ICAM-2, is not expressed on endothelium. LFA-1 (Fawcett et al., 1992) and $\alpha_D\beta_2$ (Van der Vieren et al., 1995) bind to ICAM-3, but Mac-1 and p150,95 do not (de Fougères et al., 1995).

Recently, DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN, CD209) was identified as a receptor for ICAM-3 (Geijtenbeek et al., 2000). Expression of DC-SIGN is restricted to dendritic cells (DC). It is a 44 kDa type II transmembrane protein with a 40 amino acid N-terminal cytoplasmic tail, a transmembrane domain, a region containing seven complete and one incomplete repeat sequences and a C-terminal region with homology to Ca^{2+} -dependent lectins. DC-SIGN adhesion to ICAM-3 is Ca^{2+} -dependent and can be inhibited by mannose, and, therefore, is likely to be mediated by the lectin domain. DC-SIGN, rather than the β_2 integrins, mediates DC interaction with resting T cells, which express high levels of ICAM-3. This interaction allows T cell proliferation (Geijtenbeek et al., 2000).

There is some evidence that ligation of ICAM-3 transmits signals into the cell that generate a Ca^{2+} flux and tyrosine phosphorylation of a number of proteins (Juan et al., 1994). Although tyrosine phosphorylation of ICAM-3 occurs (Skubitz et al., 1995), it is phosphorylation of Ser⁴⁸⁹, potentially by PKC- θ , that is crucial for ICAM-3 signalling (Hayflick et al., 1997).

Leukocytes become polarised upon ligation of ICAM-3 or in response to chemoattractants, such as IL-8 and fMLP. Coincidentally, there is a myosin dependent redistribution of ICAM-3 to the uropod at the rear of the cell. ICAM-3 in the uropod colocalises with the ERM protein moesin, and biochemical studies indicate that moesin interacts directly with the ICAM-3 cytoplasmic tail (Alonso-Lebrero et al., 2000; Campanero et al., 1994; Serrador et al., 1997). One function of ICAM-3 in the uropod appears to be to capture other cells. This recruitment of leukocytes provides a mechanism for enhancing transendothelial migration (del Pozo et al., 1997).

A circulating form of ICAM-3 (sICAM-3) is found in the plasma of healthy individuals at concentrations up to 360 ng/ml. These levels are elevated in inflammatory conditions. sICAM-3 is released by proteolytic cleavage of cell

surface ICAM-3 upon activation of neutrophils (del Pozo et al., 1994; Martin et al., 1995).

No information on ICAM-3 function can be obtained from knockout mice as murine ICAM-3 has not been identified. Analysis of the region of murine chromosome 9, where the ICAM genes are clustered, has failed to identify an ICAM-3 gene (Kilgannon et al., 1998).

1.2.4 ICAM-4

ICAM-4, or LW (Landsteiner-Wiener), is a 42 kDa member of the ICAM family that is expressed on erythrocytes. The two extracellular Ig-like domains of ICAM-4 have approximately 30% identity with domains 1 and 2 of ICAM-1, 2 and 3 and contain four potential N-glycosylation sites (Bailly et al., 1994). Like ICAM-1, ICAM-4 binds to LFA-1, Mac-1 and perhaps to p150,95 (Bailly et al., 1995; Hermand et al., 2000). Whether these interactions occur in vivo, however, is uncertain.

1.2.5 ICAM-5

ICAM-5, also known as telencephalin, is a 130 kDa glycoprotein expressed exclusively on neurones. It has nine Ig-like domains, with 14 potential glycosylation sites, a transmembrane region and a 61 amino acid cytoplasmic tail (Yoshihara et al., 1994). The ICAM-5 gene is located in the ICAM cluster on chromosome 19p13.2, between the ICAM-1 and ICAM-3 genes (Kilgannon et al., 1998).

Domain 1 of ICAM-5 binds to LFA-1 and it is speculated that this interaction mediates adhesion of LFA-1 expressing microglia or leukocytes with telecephalic neurones (Mizuno et al., 1997; Tian et al., 2000a; Tian et al., 1997). Unlike LFA-1 interactions with the other members of the ICAM family, LFA-1 binding to ICAM-5 is cell activation and divalent cation independent (Mizuno et al., 1997). Domain 1 of

ICAM-5 can also bind to domains 4 and 5 to promote homophilic interactions (Tian et al., 2000b). Homophilic interactions mediate binding between neurones and promote dendritic outgrowth. There is also some evidence that domain 6 of ICAM-5 binds to an as yet uncharacterised ligand (Tian et al., 2000a).

1.2.6 Structure of the ICAMs

Primary sequence analysis of ICAM-1 predicted that each extracellular domain had an immunoglobulin (Ig)-like structure with seven anti-parallel β strands (A to G) linked by a disulphide bond (Simmons et al., 1988; Staunton et al., 1988). A characteristic of the IgSF members that bind to integrins is the presence of an additional disulphide bond, which links the BC loop to the end of the F strand at the top of the domain. ICAM-1 and ICAM-3 have five Ig-like extracellular domains, ICAM-2 and ICAM-4 have two, and ICAM-5 has nine (**Fig 1.7**). These domains are numbered sequentially from the amino terminal domain. Electron microscopic studies with a soluble form of ICAM-1 composed of the five extracellular domains show that these domains are aligned in a linear fashion and have a bend approximately two fifths along the molecule, probably between domains 3 and 4 (Kirchhausen et al., 1993). This bend is not seen in ICAM-3 (Sadhu et al., 1994).

The crystal structures of domains 1 and 2 of ICAM-1 and ICAM-2 have now been solved (Bella et al., 1998; Casasnovas et al., 1997; Casasnovas et al., 1998b). The structure of domains 1 and 2 of ICAM-1 is depicted in **Figure 1.8**. Domain 1 of both ICAM-1 and 2 is a β -sandwich composed of two sheets, one containing the A', G, F and C strands and the other the A, B, E and D strands. The presence of both an A' and D β -strand means that domain 1 belongs to the Intermediate 1 (I1) class of immunoglobulin domains. Unlike the typical I-set domains, it lacks the short C' strand and instead the C strand runs directly into the CD loop that connects to the D strand. Glu³⁴ of ICAM-1 (and Glu³⁷ in ICAM-2) is critical for binding to LFA-1.



Figure 1.8 The crystal structure of domains 1 and 2 of ICAM-1

A ribbon diagram of the crystal structure of ICAM-1 domains 1 and 2. The β -strands are shown in red, α -helix in blue, the disulphide bonds in green and the N-linked carbohydrates in yellow. This figure is adapted from Casasnovas et al., 1998b.

This is the last residue in the C strand and the surrounding residues form a flat integrin binding surface, the rigidity of which is maintained by a hydrogen bonding network.

Domain 2 lacks a D strand, therefore was originally classified as a constant 2 (C2)-set Ig domain, but the presence of an A' strand has led it to be re-classified as an I2-set domain (Casasnovas et al., 1998b). ICAM-2 has an unusually long C'E loop compared to ICAM-1 and to other I2 and C2 set domains. The prominent FG loop of domain 2 cradles domain 1. In ICAM-2, hydrophobic areas at the bottom of domain 1 and the top of domain 2 are closely associated and, together with the hydrogen bonding network between the A', G and F strands in domain 1 and the FG and BC loops in domain 2, may limit interdomain flexibility. For ICAM-1, the hydrogen bonding network is more limited and, therefore, interdomain flexibility may be greater than for ICAM-2. Domain 1 is rotated 152° and has a bend of 35° in relation to domain 2 for ICAM-2, and at 170° with a bend of 30° for ICAM-1. Thus strand C in domain 1, which contains the LFA-1 binding site, is orientated away from the membrane.

ICAM-1 has no glycosylation sites in domain 1 but has four N-linked glycosylation sites in domain 2, two near the top of the domain, which may limit interdomain movement, and two near the bottom of the domain. Six N-linked glycosylation sites are present in ICAM-2, three in each domain. The three glycosylation sites in domain 1 are circumferential to the putative LFA-1 binding site around Glu³⁷. The three sites in domain 2 are described as forming a tripod that is predicted to orientate the molecule with respect to the cell surface. ICAM-3 also has glycosylation sites in domain 1. The lack of glycosylation of ICAM-1 domain 1 may therefore explain why ICAM-1 is a more promiscuous receptor than these other two members of the family.

ICAM-1 contains a dimerisation motif in its transmembrane region that partially contributes to the formation of ICAM-1 dimers on the cell surface (Reilly et al., 1995). However, it is likely that dimerisation through the extracellular domains also occurs. Consistent with this, ICAM-1 exists as a dimer in the crystal, with the face formed by the BED strands of domain 1, and, in particular Val⁵¹ in the E strand, forming the contact between the monomers. Domain 5 of ICAM-1 is also implicated in dimer formation (Miller et al., 1995). Val⁵¹ is not conserved in ICAM-2 and ICAM-2 is only a two domain molecule, which is likely to explain why ICAM-2 does not dimerise (Reilly et al., 1995). Dimerisation of ICAM-1 enhances the affinity for LFA-1, but whether this is due to the ICAM-1 dimer interacting with two sites on the same LFA-1 molecule or with two LFA-1 molecules is unknown.

1.2.7 Ligands for ICAM-1

1.2.7.a LFA-1

LFA-1, the most widely distributed member of the Leukocyte Integrin family, was the first receptor to be described for ICAM-1 (Marlin and Springer, 1987). It is also the receptor for the other members of the ICAM family. It is described in detail in **Chapter 1.1.1.a**.

1.2.7.b Mac-1

Another leukocyte integrin, Mac-1, also binds to ICAM-1 (Diamond et al., 1990). Like LFA-1, the I domain of Mac-1 contains a binding site for ICAM-1 (Diamond et al., 1993). However, LFA-1 and Mac-1 do not interact with ICAM-1 in the same way. Chimeric and domain deletion molecules indicate that domain 3 is critical for Mac-1 binding, as domains 1 and 2 can be deleted without effect

(Diamond et al., 1991). Asp²²⁹ in domain 3, which corresponds to Glu³⁴ of domain 1, is critical for binding to Mac-1 (Diamond et al., 1991).

The extent of N-linked glycosylation in ICAM-1 domain 3 is important in the regulation of Mac-1 binding. Mutation of either of the two glycosylation sites, or truncation of the sugars, enhances binding. fMLP-stimulated neutrophils bind to ICAM-1 with large carbohydrate side chains through LFA-1. However, binding to non-glycosylated forms, or those with simple side chains, is via Mac-1. Glycosylation of ICAM-1 is cell-type specific, therefore extent of glycosylation may be a method of controlling where and which leukocytes bind (Diamond et al., 1991).

Like LFA-1, Mac-1 is not constitutively active for binding to ICAM-1. Upon neutrophil activation, e.g. by fMLP, cytokines or phorbol ester, there is a rapid induction of Mac-1/ICAM-1 interaction and, occurring more slowly, but maximal by 10 minutes, a ten fold increase in cell surface expression (Miller et al., 1987).

1.2.7.c Fibrinogen

By binding to ICAM-1 on the endothelium, particularly stimulated endothelium, and either ICAM-1 or Mac-1 on the leukocyte, fibrinogen acts as a bridge to enhance adhesion between leukocytes and endothelium (Languino et al., 1993), and promotes migration of the leukocytes across the endothelium (Languino et al., 1995). Under conditions of flow this fibrinogen bridge produces firm attachment of leukocytes to the endothelium (Sriramarao et al., 1996). The interaction of ICAM-1 with fibrinogen is mediated by residues 8-21 in domain 1 of ICAM-1 binding to fibrinogen γ chain residues 117-133 (Altieri et al., 1995; D'Souza et al., 1996). In addition, two mAbs that inhibit this interaction have been mapped to residues Asp²⁶ and Pro⁷⁰ in ICAM-1 domain 1 (Duperray et al., 1997).

1.2.8 Binding of pathogens to ICAM-1

1.2.8.a *Plasmodium falciparum*

A crucial stage in malarial infections is the adhesion of erythrocytes infected with the malarial parasite *Plasmodium falciparum* to capillary endothelium. By this process, known as sequestration, the infected erythrocytes escape clearance by the spleen. The erythrocytes adhere to the endothelium for around 24 hours before they rupture and release the matured parasites into the circulation. ICAM-1 has been identified as an endothelial cell receptor for the infected erythrocytes (Berendt et al., 1989). Endothelial cell ICAM-1 expression is greatly upregulated by the cytokines, in particular TNF, which are released as part of the inflammatory response to severe malarial infections. The parasite ligand for ICAM-1 is PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), encoded by the var multigene family. PfEMP1 is a large antigenically variant protein and is expressed on the host erythrocyte surface (Baruch et al., 1996 ; Smith et al., 1995; Smith et al., 2000b).

Although there is some discrepancy between the results of the two groups who have analysed the malaria binding sites in ICAM-1 (perhaps due to the use of different *P. falciparum* clones), two regions are implicated. These regions are Gly¹⁴-Ser²² on β -strand B and Pro³⁶-Asn⁴⁷ covering the CD loop, β -strand D and the DE loop (Berendt et al., 1992; Ockenhouse et al., 1992). These residues lie on the opposite face of ICAM-1 from those affecting LFA-1 binding.

1.2.8.b Rhinovirus

Cell surface receptors for viruses serve to target the virus to specific cell types and promote entry of the viral genome into the cell. For human rhinoviruses (HRV), 90% of serotypes belong to the major group, classified with respect to their common cellular receptor, ICAM-1 (Greve et al., 1989; Staunton et al., 1989b). HRV, members of the picornavirus family, are the cause of 50% of cases of the

common cold. Picornaviruses are icosahedrons formed by 60 protomers around a single stranded RNA genome. Each protomer is composed of four capsid proteins, VP1, VP2, VP3 and VP4. The receptor binding site is located in the canyon found on each protomeric face of the virion (Olson et al., 1993). This canyon is the most conserved area on the surface of the virion and its size makes it inaccessible to the host's antibodies, therefore resistant to selective pressure from the host immune system. In vitro studies show that rhinovirus binding to a soluble form of ICAM-1 causes destabilising conformational changes in the virion, spontaneous uncoating of the virus and release of the VP4 subunit, viral RNA and ICAM-1 (Hoover-Litty and Greve, 1993).

For rhinovirus binding, the BC, DE and FG loops at the apex of ICAM-1 domain 1 penetrate into the canyon of the rhinovirus capsid, with the short CD loop sitting on the rim of the canyon (Bella et al., 1998). Mutational analysis of ICAM-1 corroborates this mode of interaction (McClelland et al., 1991; Register et al., 1991; Staunton et al., 1990).

1.3 THE ROLE OF LFA-1/ICAM-1 INTERACTIONS IN THE IMMUNE SYSTEM

1.3.1 LFA-1 and ICAM-1 as adhesion molecules

1.3.1.a Leukocyte-endothelium interactions

Leukocyte adhesion to and transmigration across endothelium are critical events in lymphocyte recirculation and leukocyte emigration into inflamed tissues. Lymphocytes continuously circulate between the blood and lymphoid tissues to survey the body for foreign antigen. This process requires the lymphocytes to move across specialised post-capillary endothelium in the high endothelial venules (HEV) of peripheral lymph nodes. In inflammatory sites bacterial products and inflammatory cytokines cause vasodilation, increase vascular permeability, and induce surface expression of adhesion molecules on the endothelial cells. These conditions initiate leukocyte migration from the blood to the inflamed tissue. A cascade of leukocyte-endothelium adhesion events mediates the transmigration out of the blood in both lymphocyte recirculation and leukocyte emigration into inflamed tissues (**Fig. 1.9**). This process has been reviewed in (Bianchi et al., 1997; Carlos and Harlan, 1994; Johnson-Léger et al., 2000) and will be discussed in brief below. During the first step, circulating leukocytes become loosely attached to the vascular endothelium and begin to roll along it. This rolling is mediated by L-selectin on the leukocyte and E- and P-selectin on the endothelium binding to sialyl-Lexis^x moieties on their glycosylated counter-receptors. The binding of the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ on the leukocyte to VCAM-1 and MAdCAM-1 on the endothelium can also support rolling but, in contrast to the selectin-mediated rolling, this requires stimulation. Chemokines, constitutively produced in lymphoid tissues or produced by cells stimulated by bacterial products or inflammatory cytokines, are found bound to

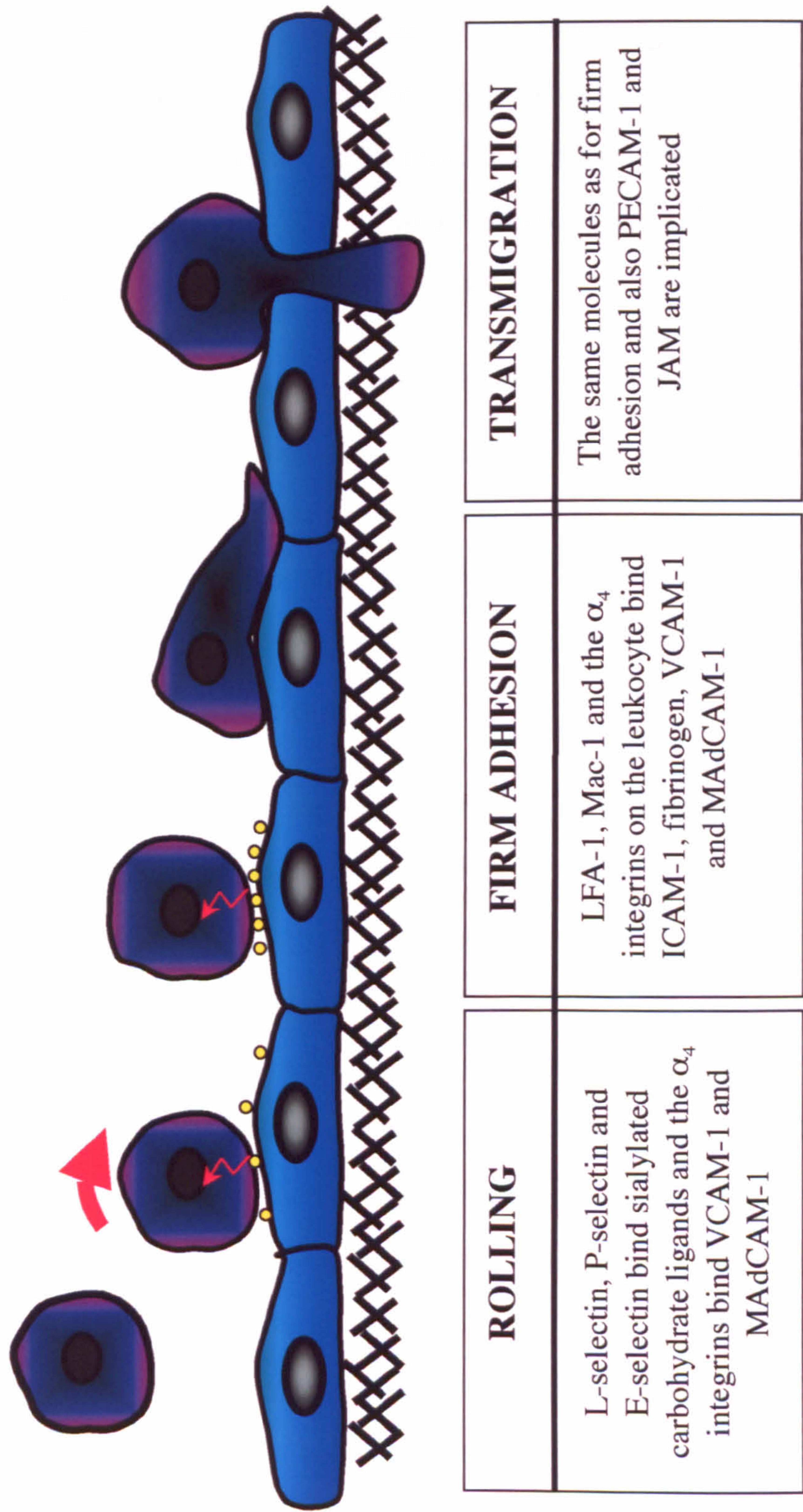


Figure 1.9 The Leukocyte Adhesion Cascade

Leukocytes roll along the endothelium using selectins and α_4 integrins. Chemokines on the endothelium (yellow circles) can trigger $\alpha_4\beta_1$ clustering and thus promote $\alpha_4\beta_1$ -mediated rolling. Further exposure to chemokines stimulates firm adhesion mediated by activation of LFA-1, Mac-1 and the α_4 integrins. The leukocytes spread and migrate on the endothelium before transmigration to the extravascular tissue.

proteoglycans on the endothelium. The chemokines activate leukocytes by engaging specific G protein-coupled receptors. Recently, it has been shown that chemokines play a role in this first phase of the adhesion cascade, rather than coming into play subsequent to rolling as previously thought (Grabovsky et al., 2000). Surface bound chemokines promote $\alpha_4\beta_1$ -mediated rolling of leukocytes on VCAM-1 by triggering extremely rapid (sub-second) clustering of $\alpha_4\beta_1$, thus increasing its avidity for VCAM-1 (Grabovsky et al., 2000).

The second step is the arrest of the leukocytes. Additional chemokine signals combined with signalling through ligated L-selectin, activate the integrins to firmly adhere to their endothelial ligands. LFA-1 is one of the integrins contributing to this stable adhesion, with Mac-1, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ also involved. The endothelial ligands are ICAM-1, fibrinogen, VCAM-1 and MAdCAM-1. The arrested leukocytes flatten and spread on the endothelium.

The next step in the process is diapedesis. This is the crawling of the leukocytes through the endothelium, directed by a gradient of chemotactic factors from extravascular sites. Diapedesis is reported to occur preferentially at points where three endothelial cells contact, as the tight and adherens junctions between the endothelial cells are weaker at these points. It is thought that leukocyte binding to the endothelial cells initiates signals that result in opening of these junctions. Proteolytic degradation of junctional proteins may also be involved at this stage. The molecules responsible for migration through the junctions are less well defined than for the earlier steps. Platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) and junctional adhesion molecule (JAM), IgSF proteins with a junctional distribution, have been implicated at this stage, with the integrins LFA-1, Mac-1, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ also predicted to be involved. Finally, the leukocytes migrate through the basement membrane and the extravascular connective tissue to the target site. This requires the proteolytic degradation of extracellular matrix (ECM) components, potentially by matrix metalloproteases, and integrin–ECM-mediated migration.

1.3.1.b Leukocyte-leukocyte interaction

As naïve lymphocytes circulate between the blood and peripheral lymph nodes, they bind transiently to antigen presenting cells (APC) until they encounter antigen. Once antigen is recognised, the T cells become activated, proliferate and differentiate into effector cells, which can respond rapidly upon subsequent antigen challenge. These effector cells are of three types: cytotoxic T cells, which kill virus infected cells; T_H1 inflammatory T cells, which activate macrophages to kill ingested bacteria; T_H2 cells, which activate B cells to produce antibody. The initial interaction of the T cell with an APC or target cell is antigen-independent and mediated by LFA-1 and CD2 on the T cell binding to ICAM-1 and LFA-3 on APC/target cell. During the initial contact phase the T cells crawl on the APC, and this is similar to what is observed when the T cells are plated on purified ICAM-1 (Dustin et al., 1997; Negulescu et al., 1996). Recognition of antigen, however, triggers an increase in adhesion so that the T cell stops and becomes more tightly bound, facilitating the stable, long-lasting interaction that is required for T cell activation. The use of purified proteins, rather than APCs, indicates that adhesion during this antigen-dependent phase can be provided by LFA-1/ICAM-1 (Dustin et al., 1997; Negulescu et al., 1996). The firm adhesion of the cells is associated with an intracellular Ca^{2+} flux followed by Ca^{2+} oscillations. Thus, LFA-1 binding to ICAM-1 plays an important role in mediating T cell interactions with APC and target cells.

1.3.2 LFA-1 and ICAM-1 as signal transduction molecules

1.3.2.a LFA-1 mediated signalling

Transmission of signals into the cell following ligand binding by integrins affects many cell processes, including migration, regulation of the actin cytoskeleton, gene expression, proliferation, differentiation and survival. Signalling events

following LFA-1 engagement are ill characterised but include stimulation of PLC γ 1 (phospholipase C γ 1) and raised intracellular Ca²⁺ levels (Kanner et al., 1993), activation of the tyrosine kinases ZAP-70 (Soede et al., 1999), Pyk-2 and p125^{FAK} (Rodríguez-Fernández et al., 1999), and translocation of Pyk-2 (Rodríguez-Fernández et al., 1999) and the β_1 and δ isoforms of PKC (Volkov et al., 1998) to the microtubule organising centre. The sequence of these events, and the consequence of each, is unknown.

1.3.2.b LFA-1 as a costimulatory molecule

Upon T cell engagement of an antigen presenting cell there is an antigen-dependent recruitment of LFA-1 to the contact point (Kupfer and Singer, 1989). With more sophisticated microscopy it has become apparent that molecules are segregated to specific areas in the cell-cell contact to form what has been termed the immunological synapse. On initial contact of the cells, a central zone of LFA-1 with a peripheral ring of TCR forms. Within minutes this structure reverses and the central area, known as the cSMAC (central supramolecular activation cluster), becomes occupied by the TCR associated with PKC- θ and the protein tyrosine kinases Fyn and Lck. This is surrounded by a peripheral area, called the pSMAC (peripheral supramolecular activation cluster), of LFA-1 colocalised with talin (Grakoui et al., 1999; Monks et al., 1998). This mature immunological synapse is stable for more than an hour and allows T cell activation. It is not clear what the precise role of LFA-1 at the contact point is.

For efficient T cell activation and proliferation to occur in response to major histocompatibility complex (MHC)-peptide, a costimulatory signal is required in addition to signalling through the TCR. CD28 is one of the best characterised costimulatory molecules for TCR signalling. Binding of B7.1 (CD80) and B7.2 (CD86) to CD28 has been shown to activate signalling pathways distinct from those

activated by TCR engagement (reviewed in (Rudd, 1996)). One mechanism by which CD28 costimulation promotes T cell activation is by recruiting lipid rafts rich in kinases to the site of TCR engagement (Viola et al., 1999). LFA-1 mediated adhesion can facilitate antigen presentation to T cells, resulting in IL-2 production and cell proliferation but, in contrast to CD28 costimulation, LFA-1 does not promote long term cell survival (Zuckerman et al., 1998). It has, however, proved difficult to identify signals specifically associated with LFA-1 engagement. It has therefore been proposed that LFA-1 does not transduce signals, but merely acts as an adhesion molecule to promote cell-cell contact, thus optimising TCR signalling by enhancing TCR engagement by MHC-peptide (Bachmann et al., 1997). One argument against this is that increasing peptide/MHC density does not restore the defects in T cell activation which occur in LFA-1 deficient T cells or when the antigen presenting cell lacks ICAM-1 (Abraham et al., 1999; Shier et al., 1999). For example, a costimulatory signal is required for IL-2 production and proliferation (Abraham et al., 1999; Geginat et al., 1999).

Prolonged LFA-1 mediated adhesion is necessary for induction of cyclin D3 and a decrease in the cyclin dependent kinase inhibitor p27^{Kip1}, which results in hyper phosphorylation of pRb and allows entry into the S phase of the cell cycle (Geginat et al., 1999). Interestingly, Jun activation domain-binding protein 1 (JAB-1), which functions as a negative regulator of p27^{Kip1} by promoting its degradation (Tomoda et al., 1999), has recently been identified as a protein that interacts with the cytoplasmic tail of the LFA-1 β_2 subunit (Bianchi et al., 2000). Whether JAB-1 is responsible for the LFA-1-induced p27^{Kip1} degradation is unknown, but LFA-1 engagement does induce JAB-1 translocation from the plasma membrane to the nucleus, where it enhances c-Jun driven transcription (Bianchi et al., 2000).

1.3.2.c Integrin crosstalk

There is increasing evidence that the function of one type of integrin can be modulated by ligation of another. This process has been termed crosstalk. ICAM-1 binding to LFA-1 alters the function of the β_1 integrins, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ on T cells (Leitinger and Hogg, 2000; Porter and Hogg, 1997) and, vice versa, engagement of $\alpha_4\beta_1$ can activate LFA-1 binding to ICAM-1 (Chan et al., 2000). The mechanism by which integrin crosstalk occurs remains unclear. It has been speculated that crosstalk may occur during transendothelial migration when leukocytes convert from a rolling to a firmly adherent phenotype.

1.3.2.d ICAM-1 mediated signalling

As well as mediating adhesion, ICAM-1 can also transmit intracellular signals. For leukocytes, ligation of ICAM-1 leads to an increase in MHC class II expression and proliferation in B cells (Gardiner and D'Souza, 1999; Holland and Owens, 1997), down-regulation of cell cycle protein kinase cdc2 activity in T cells (Chirathaworn et al., 1995), and costimulation of an oxidative burst in monocytes (Rothlein et al., 1994). The signalling pathways leading to these events are not well characterised, but involve phosphorylation of a number of proteins including the Src family tyrosine kinases p53/p56^{Lyn} and p60^{Src} and activation of the Raf-1/ERK-1 MAP-kinase cascade (Gardiner and D'Souza, 1999; Holland and Owens, 1997).

On endothelial cells, two signalling pathways initiated by ICAM-1 ligation converge to regulate organisation of the cytoskeleton. An early event is activation of PLC γ 1, which leads to elevation of intracellular Ca²⁺ levels and activation of a Ca²⁺-dependent isoform of PKC. The activation of PKC leads to phosphorylation of the tyrosine kinase p60^{Src}, which in turn phosphorylates the cytoskeletal protein cortactin and contributes to the phosphorylation of the focal adhesion associated proteins

paxillin and p125^{FAK} (Etienne-Manneville et al., 2000). The activation of the small GTPase Rho is also necessary for paxillin and p125^{FAK} (but not cortactin) phosphorylation and leads to Cas phosphorylation. Upon phosphorylation, paxillin, p125^{FAK} and Cas form a complex. Cas and paxillin associate with the adapter protein Crk that then recruits the guanine nucleotide exchange factor C3G. This pathway eventually leads to JNK activation (Etienne et al., 1998).

Both the Rho and PLC γ 1 pathways regulate cytoskeletal rearrangement and stress fibre formation. Inhibition of either pathway prevents leukocyte migration (Adamson et al., 1999; Etienne-Manneville et al., 2000). JNK (c-Jun N-terminal kinase) is a regulator of the AP-1 transcription factor complex. AP-1 mediated transcription has been observed downstream of the Erk-1 MAP-kinase pathway, rather than downstream of the JNK pathway, upon ICAM-1 engagement in endothelial cells, and is responsible for the upregulation of VCAM-1 (Lawson et al., 1999). In synovial cells, activation of AP-1 via ICAM-1 mediates transcription of IL-1 β (Koyama et al., 1996). Thus, on endothelial cells, ICAM-1 engagement, either by LFA-1-mediated leukocyte adhesion or by antibodies, results in cytoskeleton reorganisation, which is required for leukocyte diapedesis (Adamson et al., 1999), and causes upregulation of inflammatory cytokines and endothelial adhesion molecules, all of which cooperate to enhance leukocyte transmigration.

A consequence of fibrinogen binding to ICAM-1 on the endothelium is activation of the MAP kinase cascade (Pluskota and D'Souza, 2000) and phosphorylation of the phosphatase SHP-2, which associates with ICAM-1 phosphorylated on Tyr⁴⁸⁵ (Pluskota et al., 2000). These signalling pathways ultimately prevent endothelial cell apoptosis.

1.3.3 Genetic deficiencies

1.3.3.a Leukocyte Adhesion Deficiency-1

The physiological importance of the Leukocyte Integrins is revealed by an inherited syndrome known as leukocyte adhesion deficiency-1 (LAD-1). LAD-1 occurs as a result of mutations in the β_2 subunit that disrupt association with the α subunits and thereby impair transport to the cell surface. Affected individuals suffer from recurrent life-threatening bacterial infections, but mount adequate responses to viral challenge. Characteristically, LAD patients have wounds without pus formation. LAD-1 is discussed in more detail in **Chapter 6**.

1.3.3.b Knockout mice

ICAM-1 and CD18 deficient mice have similar problems as LAD-1 patients. ICAM-1 deficient mice have elevated peripheral blood neutrophil counts, impaired neutrophil infiltration into inflamed tissue, suppressed contact hypersensitivity, and indicate a critical role for ICAM-1 on stimulator cells, but not on responder cells, in mixed lymphocyte reactions (Sligh et al., 1993).

Mice deficient in the leukocyte integrin β_2 subunit (CD18) also exhibit elevated peripheral blood neutrophil counts, have splenomegaly and lymphadenopathy, and develop spontaneous skin lesions. Neutrophil migration into these lesions or to induced inflamed skin is severely impaired (Mizgerd et al., 1997; Scharffetter-Kochanek et al., 1998). Neutrophil extravasation in response to lung inflammation or peritonitis is, however, normal (Mizgerd et al., 1997). Functional abnormalities in T cells are also apparent. T cells from these mice do not proliferate in response to staphylococcal enterotoxin or MHC alloantigens (Scharffetter-Kochanek et al., 1998). In addition, CD18 deficient mice have almost complete

absence of intraepithelial lymphocytes, a subset of T cells found in intestinal epithelium (Huleatt and Lefrançois, 1996).

These studies demonstrate that the leukocyte integrins are responsible for leukocyte migration to some sites of inflammation and play a role in some T cell responses. The specific contribution of each member of this family of integrins was, however, unclear until the development of LFA-1 and Mac-1 knockout mice. LFA-1 deficient mice have small peripheral lymph nodes and reduced migration into lymph nodes (Berlin-Rufenach et al., 1999; Schmits et al., 1996), reflecting the essential role of LFA-1 in mediating T cell interaction with HEV of lymph nodes and Peyer's patches (Berlin-Rufenach et al., 1999; Warnock et al., 1998). Thus LFA-1 is important for lymphocyte homing.

Lymphocytes from LFA-1 null mice fail to aggregate and have impaired proliferation in mixed lymphocyte reactions and in response to the mitogen concavalin A (Schmits et al., 1996; Shier et al., 1996). Proliferative responses in response to lipopolysaccharide, triggering the T cell receptor CD3 subunit, or activating the cells with phorbol ester and ionomycin (to activate PKC and raise intracellular Ca^{2+} concentration) were all found to be normal (Shier et al., 1996). CTL function in response to virus was not affected by absence of LFA-1 (Schmits et al., 1996).

Surprisingly, comparison of LFA-1 and Mac-1 knockout mice demonstrated a dominant role for LFA-1 in neutrophil recruitment to inflammatory sites and for neutrophil adhesion to endothelial cells and to purified ICAM-1 (Ding et al., 1999; Lu et al., 1997). The specific roles of Mac-1 are revealed by the inability of Mac-1 deficient neutrophils to phagocytose iC3b coated particles, degranulate or produce a respiratory burst (Lu et al., 1997).

1.4 AIMS

The major aim of this thesis was to increase the understanding of how the leukocyte integrin LFA-1 interacts with its ligands ICAM-1 and ICAM-3. On the ICAM side of the interaction, this involved identifying residues in ICAM-1 and ICAM-3 that are important for binding LFA-1. For LFA-1, the objectives were to discover what alterations occur to form the high affinity LFA-1 conformation and to understand how intracellular signalling pathways induce LFA-1 adhesion without increasing its affinity for ICAM-1. The opportunity arose to analyse the dysfunction in leukocytes from a unique patient with the characteristic symptoms of Leukocyte Adhesion Deficiency.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Details of companies can be found in Appendix III

2.1.1 Stimulants , inhibitors and other reagents

Aprotonin	Sigma	A 2.2 mg/ml solution. Store at 4°C
BCECF-AM (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester)	Calbiochem	Stock 1 mM in dimethyl sulphoxide (DMSO). Store at -20°C
Calpeptin	Calbiochem	Stock 10 mg/ml in DMSO. Store at -20°C
CBZ-LVG (CBZ-Leu-Val-Gly diazomethyl ketone)	Sigma	Stock 10 mM in DMSO. Store at -20°C
DHR (di-hydrorhodamine)	Cambridge Bioscience	Stock 10 mM in DMSO. Store at -20°C
fMLP (N-formyl-methionyl-leucyl-phenylalanine)	Sigma	Stock 10 mM in ethanol. Store at -20°C
Ionomycin	Calbiochem	Stock 1 mM in DMSO. Store at 4°C
Jasplakinolide	Molecular Probes	Stock 1 mM in DMSO Store at -20°C
Lactacystin	Calbiochem	Stock 10 mM in DMSO Store at -20°C
PdBu (Phorbol-12,13-dibutyrate)	Calbiochem	Stock 2 mM in DMSO. Store at -20°C
PMSF (Phenylmethanesulphonyl fluoride)	Sigma	Stock 10 mg/ml in ethanol. Make fresh each time.
SKF-96365	Calbiochem	Stock 50 mM in H ₂ O Store at -70°C
Thapsigargin	Calbiochem	Stock 2 mM in DMSO. Store at -20°C

2.1.2 Buffers

2.1.2.a HEPES buffer

20 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]),
140 mM NaCl, 2 mg/ml glucose, adjusted to pH 7.4

2.1.2.b H-HBSS

Hanks Balanced Salt Solution (HBSS; 10 x; Gibco-BRL) containing 10 mM
HEPES (1 M; Sigma)

2.1.2.c 0.1 M Carbonate buffer pH 9

1.36 g/l Na_2CO_3 , 7.35 g/l NaHCO_3

2.1.2.d FACSwash

Phosphate-buffered saline (PBS)-A containing 0.2% bovine serum albumin
(BSA; Sigma) and 0.1% sodium azide

2.1.2.e FACSfix

PBS-A containing 2% formaldehyde

2.1.2.f Other stock solutions

Roosevelt Park Memorial Institute medium (RPMI 1640), Dulbecco's
modified Eagle's medium (DMEM) and PBS-A (1x and 10x) were prepared by
Central Cell Services, ICRF.

2.1.3 Antibodies

2.1.3.a ICAM-1 mAbs

ICAM-1 mAbs used in this study were: 15.2 (Research Monoclonal Antibody Services, ICRF), My13 (Curt Civin, Baltimore, USA), 7F7 (T. Schulz, Munich, Germany), RR1/1 and R6.5 (R. Rothlein, Ridgefield, CT, USA), WEHI-CAM-1 (A. Boyd, Melbourne, Australia), 7.5C2 and 8.4A6 (D. Haskard, London), CBR-IC1/11 and CBR-1C1/12 (Leukocyte Typing V).

2.1.3.b ICAM-3 mAbs

ICAM-3 mAbs used in this study were: BRIC79, CG106, CBR-1C3/1, CBR-1C3/2, CBR-1C3/3, CBR-1C3/4, CBR-1C3/5, CBR-1C3/6, WDS 3A9, BY44, TP1/24, HP2/19, KS128, 152-2D11, 140-11, 101-1D2 (all from Leukocyte Typing V), CH3.1, CH3.2 and CH3.3 (D. Simmons, Oxford), CG106, BY44, KS118 and KS128 (D. Mason, Oxford).

2.1.3.c Leukocyte integrin mAbs

LFA-1, Mac-1 and p150,95 mAbs used in this study were: 38 (CD11a), ICRF44 (CD11b), 3.9 (CD11c), 24 (activation epitope on β_2 integrins) all obtained from Research Monoclonal Antibody Services, ICRF. Also KIM185 (β_2 activating mAb; M. Robinson, Celltech, Slough), S6F1 (CD11a; C. Morimoto, Boston, USA), G25.2 (CD11a; Becton Dickinson), TS2/4 (CD11a; American Type Culture Collection), IB4 (CD18; A. Law, Oxford) and TS1/18 (CD18; American Type Culture Collection).

2.1.3.d Other integrin mAbs

Other integrin mAbs used were: HP2/1 (CD49d; Serotec), SAM-1 (CD49e; Eurogenetics), P5D2 (CD29; American Type Culture Collection), HUTS21 (activation epitope on β_1 integrins; C. Cabañas, Madrid, Spain), 15/7 (activation epitope on β_1 integrins; T. Yednock, Elan Pharmaceuticals, San Francisco, USA) and TS2/16 (β_1 activating mAb; C. Cabañas, Madrid, Spain)

2.1.3.e Antibodies against cytoskeletal and signalling proteins

The cytoskeletal and signalling protein antibodies used were against talin (clone 8d4), α -actinin (clone BM-75.2), vinculin (clone hVIN-1), ezrin (clone 3C12), all purchased from Sigma, and filamin, purchased from Chemicon. All were used at 1/5000 for Western blotting. Also cytohesin-1 clone 10 (1/10 dilution of supernatant; W. Kolanus, Munich, Germany), Pyk-2 (1/5000; Santa Cruz).

2.1.3.f Other primary mAbs

Other antibodies used were: 52U (IgG1 control), 4U (IgG2a control), UCHT1 (CD3), DS4.20 (CD4), 14 (CD8), UCHM1 (CD14), 3G8 (CD16), BU12 (CD19), 2D1 (CD45RA), UCHL1 (CD45RO) all from Research Monoclonal Antibody Services, ICRF; LAM1.3 (L-selectin; T. Tedder, Duke University, NC, USA) and G19.4 (CD3; Bristol-Myers Squibb, Seattle, USA)

2.1.3.g Secondary antibodies

Conjugated secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (Ig) (Dako) used at 1/5000 for Western blotting; HRP-conjugated sheep anti-mouse Ig (Amersham Pharmacia Biotech) used at 1/50,000 for Western blotting; HRP-conjugated rabbit anti-goat IgG (Sigma) used at

1/5000 for Western blotting; HRP-conjugated goat anti-rat Ig (Southern Biotechnology Associates Inc.) used at 1/10,000 for Western blotting; HRP-conjugated goat anti-mouse Ig (Dako) used at 1/500 for enzyme-linked immunosorbent assays (ELISA); fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Sigma) used at 1/400 for flow cytometry; FITC-conjugated goat anti-mouse IgG Fc (Jackson ImmunoResearch Labs.) used at 10 µg/ml for confocal microscopy.

2.1.4 Recombinant proteins

2.1.4.a ICAM-1Fc (three domain)

A plasmid encoding the N-terminal three domains of ICAM-1 fused to the hinge, CH2 and CH3 domains of human IgG₁ was obtained from D. Simmons, Oxford. This construct consisted of the CDM8 vector into which the sequence encoding the three extracellular domains of ICAM-1 was cloned in frame with a human IgG₁ Fc sequence in which a mutation had been made to prevent di-sulphide bond formation. ICAM-1Fc (three domains) protein was expressed by transient transfection of COS-1 cells as described in **Chapter 2.2.1**.

2.1.4.b ICAM-1Fc (five domain)

A plasmid encoding the five extracellular domains of ICAM-1 fused at the C-terminus to the hinge, CH2 and CH3 domains of human IgG₁ (ICAM-1Fc) was obtained from A. Craig, Oxford. This construct consisted of the CDM8 based expression vector pIG1 into which a sequence encoding the leader sequence and all five extracellular domains of ICAM-1 was cloned in frame with the human IgG₁ Fc sequence. ICAM-1Fc (five domain) was produced by transient transfection of COS-1 cells with this plasmid as described in **Chapter 2.2.1**.

2.1.4.c ICAM-1Fc domain 2 mutants

Plasmids encoding mutant versions of the ICAM-1Fc constructs were provided by P. Stanley, Leukocyte Adhesion Laboratory, ICRF. Mutations were introduced by site directed mutagenesis using standard protocols. Nomenclature for mutants identifies the wild type (wt) amino acid, its position within the protein, and a forward slash followed by the amino acid substitution. The mutant proteins were produced by transient transfection of COS-1 cells as described in **Chapter 2.2.1**.

2.1.4.d K²⁹/M ICAM-1Fc (ICAM-1^{Kilif})

A five domain version of ICAM-1Fc (the five extracellular domains of ICAM-1 fused at the C-terminal to the Fc portion of human IgG₁) with the single substitution of a methionine for a lysine at position 29 (Craig et al., 2000) was provided by A. Craig, Oxford.

2.1.4.e ICAM-3Fc and mutants

ICAM-3Fc was produced by transient transfection of COS-1 cells (see **Chapter 2.2.1**) with the Fc expression vector pIG1 encoding the two N-terminal domains of ICAM-3 (provided by D. Simmons, Oxford) (Holness et al., 1995). Alternatively, ICAM-3Fc and mutant forms of ICAM-3Fc were obtained as purified proteins from D. Simmons, Oxford.

2.1.4.f VCAM-1Fc

VCAM-1Fc, consisting of the two N-terminal domains of VCAM-1 fused to the Fc fragment of human IgG₁ was obtained from M. Robinson, Celltech, Slough as a purified protein.

2.1.4.g I-Fc

A plasmid encoding the LFA-1 I domain fused to the Fc portion of human IgG₁ (I-Fc) was obtained from A. Randi, Leukocyte Adhesion Laboratory, ICRF. This consisted of the CDM8 based expression vector pIG1 into which a sequence encoding the 25 amino acids of LFA-1 signal peptide, the first four amino acids of the mature subunit, amino acids Gly¹²⁸-Ser³²⁷ of the I domain and the first 11 amino acids of domain 3 had been cloned in frame with the IgG₁ Fc sequence (Randi and Hogg, 1994). I-Fc was produced by transient transfection of COS-1 cells as described in Chapter 2.2.1.

2.1.4.h I-GST

Escherichia coli TOPP 2 transformed with the pGEX2T vector encoding the LFA-1 residues Leu¹¹¹-Ser³²⁷, which include the predicted I domain sequence and a 17 amino acid N-terminal extension, fused to glutathione S-transferase (GST) were provided by P. Stanley, Leukocyte Adhesion Laboratory, ICRF (Stanley and Hogg, 1998). These were grown to log phase and induced with 0.1 M isopropyl-1-thio- β -D-galactopyranoside (IPTG; Amersham Pharmacia Biotech) for 4 hours. The LFA-1 I domain fused to glutathione S-transferase (I-GST) was extracted from the bacterial pellets using the GST purification module kit (Amersham Pharmacia Biotech) following manufacturer's instructions.

2.1.5 Peptides

Peptides spanning the LFA-1 I domain were synthesised by the Peptide Synthesis Service, ICRF on a model 430A Applied Biosystems solid phase synthesiser using 9-fluorenyl-methoxycarbonyl chemistry and characterised by amino acid analysis and plasma desorption spectrometry. A peak corresponding to the expected molecular weight was found for each peptide and in all cases contained

the correct sequence. Peptides were desalted using Sephadex G10 (Amersham Pharmacia Biotech) and stored as a lyophilised powder at room temperature (RT) under desiccating conditions. All peptides were tested for cytotoxic activity using a MTT cytotoxicity assay (see **Chapter 2.2.10**) and were used in experiments at concentrations at which they were fully soluble.

2.2 METHODS

2.2.1 Transient transfection of COS-1 cells

Transient transfection of COS-1 cells with Fc chimeric constructs was facilitated by either diethylaminoethyl-dextran (DEAE dextran), which is thought to bind to the cells and promote endocytosis of the DNA, or electroporation, which transiently permeabilises the cells with an electrical pulse, allowing uptake of the DNA.

2.2.1.a DEAE dextran

15 cm tissue culture dishes of confluent COS-1 cells were incubated for 4 hours at 37°C with 20 ml DMEM containing 30 µg DNA, 400 µg/ml DEAE dextran (Sigma) and 60 µg/ml chloroquine (Sigma). The plates were then rinsed with ice cold 10% DMSO/PBS-A and left overnight in DMEM/10% foetal calf serum (FCS). The following day the cells were split 1 in 2 and 24 hours later washed into serum free DMEM. After 5 days the tissue culture medium was collected and the secreted protein purified as described in **Chapter 2.2.3**.

2.2.1.b Electroporation

Confluent COS-1 cells were removed from 15 cm tissue culture dishes with trypsin/EDTA, washed in RPMI-1640 and resuspended in 500 µl RPMI-1640 containing 30 µg DNA. These cells were placed in an electroporation cuvette with a 4 mm electrode gap and subjected to a voltage of 250 V for 4-6 milliseconds using a Gene Pulser (Bio-Rad) connected to a Capacitance Extender (Bio-Rad) set at 250 µF. The cells were then re-plated on 15 cm tissue culture dishes in DMEM/10% FCS for 24 hours before washing into serum free DMEM. After 5 days the tissue culture

medium was collected and the secreted protein purified as described in **Chapter 2.2.3.**

2.2.2 ICAM-1Fc preparation from CHO cells

CHO cells were stably transfected with a chimeric construct composed of the five extracellular domains of ICAM-1 fused to the Fc tail of human IgG₁ (ICAM-1-Fc) by P. Stanley, Leukocyte Adhesion Laboratory, ICRF. These were grown in supplemented Glasgow minimum essential medium (GMEM-S; see **Appendix II**) with 10% FCS and selected with 100 μ M methionine sulfoxamine (Sigma). The cells were expanded every two days until 30-40 175 cm² flasks were obtained. On the day after the final expansion the cells were washed into serum free GMEM-S supplemented with 2 mM sodium butyrate (Sigma) to enhance protein secretion. After 7-10 days the cell culture supernatant was collected and the secreted protein purified by affinity chromatography as described in **Chapter 2.2.3.**

2.2.3 Fc chimeric protein purification

Particulate matter was removed from the cell culture supernatant by centrifugation at 1300 x g for 10 minutes (min) then filtration through a 0.22 μ m filter unit (Millipore). The supernatant was then loaded onto a Protein A Sepharose (Amersham Pharmacia Biotech) column with a flow rate of 1 ml/min. Once all the supernatant was loaded, the column was washed with 30 ml of PBS-A to remove unbound material. The Fc chimeric protein was eluted with 0.1 M citrate buffer pH 3 and collected in 2 ml fractions, which were immediately neutralised with 500 μ l of 1 M Tris pH 9. The fractions containing protein, identified by absorbance at 280 nm measured on an Ultraspec III (Amersham Pharmacia Biotech), were pooled, dialysed into PBS-A and, if necessary, concentrated using Centricon centrifugal filter devices (Millipore). The integrity and purity of the protein was checked by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis; see **Chapter 2.2.19**)

and the concentration estimated using the BCA Protein Assay Reagent (Pierce) as per manufacturers instructions.

2.2.4 ^{35}S -methionine labelling

Confluent COS-1 cells, grown in 6 well tissue culture plates, were incubated for 4 hours at 37°C with 500 µl/well DMEM containing 5 µg DNA, 400 µg/ml DEAE dextran (Sigma) and 60 µg/ml chloroquine (Sigma). The wells were then rinsed with ice cold 10% DMSO/PBS-A and left overnight in DMEM/10% FCS. The following day the cells were rinsed in methionine free DMEM, incubated at 37°C for 15 min in methionine free DMEM, then incubated at 37°C for a further 5 hours in 500 µl/well methionine free DMEM supplemented with 100 µCi ^{35}S -methionine (Amersham Pharmacia Biotech). After this time the cell culture supernatant was collected, microfuged for 10 min at 4°C to remove particulate material, and 1 µg/ml aprotonin and 20 µg/ml PMSF added. The cells were lysed on the plates by addition of 500 µl/well of lysis buffer (50 mM Tris pH 8, 1% Triton X-100 (t-octylphenoxypolyethoxyethanol), 1 µg/ml aprotonin and 20 µg/ml PMSF) for 30 min on ice. The cell lysates were then microfuged for 20 min at 4°C to remove insoluble material. The supernatants and lysates were precleared with Sepharose CL4B (Amersham Pharmacia Biotech) for 3 hours at 4°C, then the Fc chimeric proteins were affinity purified overnight at 4°C with 200 µl of a 50% slurry of Protein A Sepharose (Amersham Pharmacia Biotech). The sepharose beads were washed four times, alternating high salt (500 mM NaCl) and low salt (150 mM NaCl) containing lysis buffer, and finally in lysis buffer without salt. The beads were boiled for 5 min in SDS-PAGE sample buffer and proteins separated by SDS-PAGE on 7.5% gels (see **Chapter 2.2.19**). ^{35}S -methionine labelled proteins were detected by exposure to film.

2.2.5 ELISA

Immulon-1 96 well plates (Dynex) were coated overnight at 4°C with 50 µl/well ICAM-1Fc, ICAM-3Fc (or mutated versions of these proteins), at 5 µg/ml in PBS-A (unless indicated otherwise). Unbound protein was removed and the plates blocked with 150 µl/well 2% FCS/PBS-A for 1 hour at RT. The wells were washed three times with PBS-A prior to addition of 50 µl/well of primary mAb for 30 min at RT (mAb diluted in 2% FCS/PBS-A at 10 µg/ml, unless otherwise indicated), and again before addition of 50 µl/well HRP-conjugated goat anti-mouse Ig (Dako) diluted in 2% FCS/PBS-A. Unbound antibody was removed by washing as above and bound antibody detected with 75 µl/well o-phenylenediamine dihydrochloride (OPD) detection buffer (20 ml 0.1 M NaHPO₄, 10 ml 0.1 M citric acid, 10 µl H₂O₂ plus one OPD tablet (Sigma)). The reaction was stopped with 50 µl/well 3 M H₂SO₄ and the absorbance at 492 nm read on a Titertek Multiskan plate reader.

2.2.6 T cell purification and culture

Blood from a buffy coat (National Blood Service) was diluted 1 in 4 with RPMI-1640. 60 ml of the diluted blood were layered over 30 ml of Lymphoprep (Nycomed) in 100ml glass tubes then centrifuged at 1380 x g for 30 min at RT. Peripheral blood mononuclear cells (PBMC) were removed from the plasma/Lymphoprep interface. The harvested cells were washed three times in RPMI-1640 then cultured in RPMI-1640 + 10% FCS + 1 µg/ml phytohaemagglutinin (PHA) (Murex Biotech Ltd.). After 3 days the cells were washed three times in RPMI-1640 and maintained at approximately 10⁶ cells/ml in RPMI-1640 + 10% FCS + 20 ng/ml recombinant interleukin-2 (IL-2) (Chiron UK Ltd.) for 7 - 14 days (Cantrell and Smith, 1983).

2.2.7 Purification of neutrophils

Neutrophils were isolated from EDTA or heparin anticoagulated venous blood as described by Dooley et al. (Dooley et al., 1982). Erythrocytes were sedimented by the addition of 1 ml of 6% Dextran T500 (Amersham Pharmacia Biotech) in 150 mM NaCl per 10 ml of blood. After 45 min at RT the leukocyte-rich fraction was layered onto a discontinuous 70%/80% isotonic Percoll (Amersham Pharmacia Biotech) gradient and centrifuged at 1000 x g for 15 min at RT. The neutrophil fraction was harvested from the interface between the 70% and 80% Percoll and washed three times in H-HBSS.

2.2.8 T cell adhesion assays

Immulon-1 96 well plates (Dynex) were coated overnight at 4°C with 50 µl/well ICAM-1Fc, ICAM-3Fc (or mutated versions of these proteins), VCAM-1Fc (all at 5 µg/ml unless indicated otherwise) or fibronectin (Sigma) at 20 µg/ml, all diluted in PBS-A. Unbound protein was removed and the plates blocked with 150 µl/well 2.5% BSA/PBS-A for 1 hour at RT. The wells were washed three times with assay buffer.

T cells, washed and resuspended at 4×10^6 /ml in HEPES buffer, were labelled for 30 min at 37°C with 2.5 µM BCECF-AM. Cells were then washed into the appropriate assay buffer and added to the coated plate at a final concentration of 2×10^5 cells/100 µl in the presence or absence of stimuli and inhibitors as indicated.

Plates were spun at 66 x g for 1 min then incubated at 37°C for 30 min. Unbound cells were washed off with warm HEPES buffer containing 0.4 mM MgCl₂ and 0.4 mM CaCl₂. Bound cells were quantified using a Cytofluor Multi-well Plate Reader Series 4000 (PerSeptive Biosystems) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.2.9 Neutrophil binding to fibrinogen

A 0.5 mg/ml solution of fibrinogen (Sigma) was prepared in 0.1 M carbonate pH 9 by incubating at 37°C for 30 min then filtering through a 0.2 µm syringe filter. Maxisorp 96 well Nunc-Immunoplates (Gibco-BRL) were coated overnight at 4°C with 50 µl/well of the fibrinogen solution. Unbound protein was removed and the plates blocked with 150 µl/well 2% BSA/PBS-A for 1 hour at RT. The wells were washed three times with H-HBSS.

Neutrophils, washed and resuspended at 2×10^6 /ml in H-HBSS, were labelled for 30 min at RT with 1 µM BCECF-AM. After washing in H-HBSS the cells were added to the fibrinogen coated plate at a final concentration of 10^5 cells/100 µl in H-HBSS with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ together with stimulating or blocking agents as indicated in the figure legends. Plates were incubated at RT for 30 min. Unbound cells were removed by washing the plates with H-HBSS and the number of bound cells quantified using a Cytofluor Multi-well Plate Reader Series 4000 (PerSeptive Biosystems) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.2.10 MTT cytotoxicity assay

To test the viability of cells following treatment with peptides and inhibitors MTT (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) cytotoxicity assays were performed based on the method of Denizot and Lang (Denizot and Lang, 1986). This assay is based on the ability of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water soluble substrate MTT into a dark blue water insoluble product. Cells were incubated with the relevant concentration of peptide or inhibitor plus 0.5 mg/ml MTT (Sigma) in a 96 well plate for 3 hours at 37°C. Cells without peptide or inhibitor were included as a positive control and 50 µM digitonin added as a negative control. Each condition was performed in triplicate. The reaction was stopped by the addition of 150 µl/well

of 40 mM HCl in isopropanol and the plates left in the dark overnight. The absorbance at 570-630 nm was then read on a Titertek Multiskan plate reader. Cell viability was calculated as a percentage of the absorbance of untreated cells after the negative control had been subtracted.

2.2.11 Flow cytometry

5×10^5 cells were incubated in 50 μ l of FACSwash containing primary antibody (including peptides or stimulatory agents where indicated) for 30 min on ice, or at 37°C where indicated. The cells were then washed three times in FACSwash and resuspended in 50 μ l of FACSwash containing a 1:400 dilution of FITC-conjugated goat anti-mouse IgG (Sigma). After 30 min on ice the cells were washed three more times in FACSwash, resuspended in 200 μ l of FACSfix and analysed using a FACScan (Becton Dickinson).

2.2.12 Antibody staining for confocal microscopy

For immunofluorescence analysis by confocal microscopy, 13 mm round glass coverslips were precoated with a 0.01% solution of poly-L-lysine (Sigma) for 10 min at RT, washed twice in RPMI-1640 then left to air dry. T cells were washed three times in RPMI-1640 before addition onto coverslips (5×10^5 cells/coverslip), in the presence of stimulants and CD11a mAbs at 10 μ g/ml. Coverslips were spun at 40 x g then incubated for 30 min at 37°C. Unbound cells were removed by gentle washing in warm RPMI-1640. In order to prevent antibody-induced clusters, cells were fixed with 1% formaldehyde in PBS-A for 10 min at RT, prior to a second incubation with 10 μ g/ml FITC-conjugated goat anti-mouse IgG Fc (Jackson ImmunoResearch Labs) for 25 min at 4°C.

Cells were mounted for confocal microscopy, which was carried out using an MRC-600 Confocal Laser Scanning System (Bio-Rad Laboratories Ltd.). The

regions of the confocal microscopy images with the highest fluorescence intensity (i.e. with the pixel colour intensity in the 150-255 range) were highlighted in red using the Segmentation utility of the IP Lab Spectrum Version 3.1 software (Scanalytics).

2.2.13 Analysis of clustering

IP Lab Spectrum Version 3.1 software was also used to quantify the levels of fluorescence of the images. Background fluorescence was estimated by measuring the signal strength in areas visibly devoid of specific staining but in close proximity to cells. The average of these values was then subtracted from the original image to give a corrected image. The areas of interest (e.g. free membrane or membrane at cell-cell contact points) on the corrected images were selected and the average signal strength calculated automatically by the computer software. A statistical assessment of the differences between the treatment groups and the untreated resting T cells was performed using the one-way ANOVA test.

2.2.14 Measurement of intracellular calcium ($[Ca^{2+}]_i$)

Cells were incubated at 10^7 /ml in H-HBSS with 5 μ M Fluo-3 (Molecular Probes) at 37°C for 30 min. After three washes the cells were resuspended at 2×10^6 /ml in H-HBSS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells were kept at 37°C and 2 ml aliquots added to quartz fluorimeter cuvetts (Sigma). Stimulants were added at the indicated time points and the samples stirred constantly. Fluorescence was monitored to determine $[Ca^{2+}]_i$ using a Photon Technologies International fluorimeter with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Results are represented as the emission at 530 nm.

2.2.15 Measurement of neutrophil respiratory burst

The production of superoxide was measured according to the method of Smith and Weidemann (Smith and Weidemann, 1993). Neutrophils at $2 \times 10^6/\text{ml}$ were labelled with $1 \mu\text{M}$ DHR for 5 min at 37°C prior to exposure to stimuli. After 30 min stimulation at 37°C the samples were transferred to ice. The generation of superoxide was measured by flow cytometry using a FACScan.

2.2.16 Chemotaxis assays

Migration assays were performed in 24 well Transwells with a 6.5 mm diameter and $5 \mu\text{m}$ pores (Corning Costar) that had been pre-incubated in RPMI-1640 for 1 hour at 37°C . The bottom well was filled with 600 μl of human stromal cell-derived factor (SDF)-1- α (PeproTech) at the indicated concentration in RPMI-1640 with 0.5% BSA and 100 μl of T cells at $5 \times 10^6/\text{ml}$ in RPMI-1640 with 0.5% BSA were placed in the top well. Migration was carried out at 37°C for 90 min after which the top well was discarded, the cells in the bottom well collected, and the wells washed with ice cold PBS-A containing 5 mM EDTA to collect any adherent cells. The cells were pelleted and resuspended in 200 μl of FACSwash. Cells were counted for 20 seconds using a FACScan, with a known number of CaliBRITE 3.I beads (Becton Dickinson) as a standard. The number of cells in the starting population and the number of cells which accumulated in the bottom well were calculated as follows:

$$\frac{\text{no. of cells counted} \times \text{total no. of beads added}}{\text{no. of beads counted}}$$

2.2.17 Preparation of detergent soluble T cell extracts

T cells were suspended at $5 \times 10^7/\text{ml}$ in ice cold lysis buffer (50 mM Tris pH8 containing 150 mM NaCl, 2 mM MgCl_2 , 2 mM EGTA, 1 $\mu\text{g}/\text{ml}$ aprotinin, 20 $\mu\text{g}/\text{ml}$

PMSF and 1% Triton X-100) and lysed for 20 min on ice. The lysate was then microfuged for 15 min to remove insoluble material. The soluble material was either immediately used for immunoprecipitation or mixed with an equal volume of SDS-PAGE sample buffer (see **Chapter 2.2.19**) and stored at -20°C for use as total cell lysate in Western blotting

2.2.18 Immunoprecipitation

Immunoprecipitates were prepared from the Triton X-100-soluble cell fraction which had been precleared for 1 hour at 4°C by rotation with 50 μl of a 50% slurry of Protein A Sepharose (Amersham Pharmacia Biotech) per 1 ml of lysate. LFA-1 was immunoprecipitated from the precleared lysates with 10 $\mu\text{g/ml}$ mAb 38 and 50 μl of 50% Protein A Sepharose by rotating overnight at 4°C . To remove non-specifically bound material the immunoprecipitates were washed five times in lysis buffer. Precipitated material was removed from the Protein A Sepharose beads by boiling for 5 min in SDS-PAGE sample buffer (see **Chapter 2.2.19**) and the beads were then pelleted by centrifugation and removed.

2.2.19 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli et al (Laemmli, 1970). Proteins were separated on polyacrylamide gels of the indicated percentage prepared from a 30% acrylamide/0.8% bis-acrylamide stock solution (Anachem) in 375 mM Tris pH 8.8 containing 0.1% sodium dodecyl sulphate (SDS) and polymerised with 0.04% ammonium persulphate (APS) and 1/500 N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma). The separating gels were overlaid with a 3% polyacrylamide stacking gel prepared from the acrylamide/bis-acrylamide stock solution in 125 mM Tris pH 6.8 containing 0.1% SDS and polymerised with APS and TEMED as above. Proteins for electrophoresis were boiled for 5 min in sample buffer (125 mM Tris pH6.8, 25% glycerol, 2% SDS, 0.02% bromophenol

blue), containing 1% 2-mercaptoethanol (Sigma) when reducing conditions were required, before loading onto the gels. Rainbow coloured protein molecular weight markers in the range 14.3 kDa-220 kDa (Amersham Pharmacia Biotech) were run on each gel as standards. Electrophoresis was carried out in Atto Dual Mini Slab Chamber (Genetic Research Instrumentation Ltd.) containing electrophoresis running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) at 90 volts through the stacking gel and 180 volts through the separating gel. When required, proteins were visualised by staining with Coomassie Blue (0.5% Coomassie Blue (Bio-Rad) in 40% ethanol and 10% glacial acetic acid) and destained with a solution of 20% ethanol and 10% glacial acetic acid.

2.2.20 Western blotting

Following separation by SDS-PAGE, proteins were transferred onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech) in a Transblot Cell (Bio-Rad) using Transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol). Proteins were transferred for 75 min at 60 volts. Proteins were visualised, to ensure even transfer had taken place, by staining the membrane with 0.1% Ponceau S Solution (Sigma) for a few minutes. To minimise non-specific antibody binding, membranes were blocked overnight at 4°C in 5% milk powder in PBS-A/0.1% polyoxyethylenesorbitan monolaurate (Tween 20). The membranes were incubated for 1 hour at RT with the primary antibody diluted in PBS-A/0.1% Tween 20 followed by a 1 hour incubation at RT with the appropriate HRP-conjugated secondary antibody diluted in PBS-A/0.1% Tween 20. After each antibody incubation the membranes were washed three times for 5 min with PBS-A/0.1% Tween 20. Bound antibody was detected by chemiluminescence using ECL Western blotting detection reagents (Amersham Pharmacia Biotech) as per manufacturer's instructions.

CHAPTER 3

LFA-1 BINDING SITES ON ICAM-1 AND ICAM-3

3.1 INTRODUCTION

The interaction of the leukocyte integrin LFA-1 with its ligands ICAM-1, ICAM-2 and ICAM-3 is a central feature of the immune response. Many integrins recognise the sequence Arg-Gly-Asp (RGD) in their ligands. The ICAMs, however, do not possess an RGD sequence (Fawcett et al., 1992; Staunton et al., 1989a; Staunton et al., 1988), and thus the manner in which they are bound by the integrins has been of interest. This chapter describes three projects in which I have participated that have contributed to the characterisation of LFA-1 binding sites in ICAM-1 and ICAM-3. The introduction will cover aspects of ICAM-1 and ICAM-3 relating to these projects.

All ICAM-1 mAbs that disrupt interaction with LFA-1 map to the two N-terminal domains, and a domain 1 and 2 truncated version of ICAM-1 is sufficient for LFA-1 binding (Berendt et al., 1992; Staunton et al., 1990). Mutation of residues Asp²⁶-Leu³⁰, Lys⁴⁰, Leu⁴³-Asn⁴⁷ and Pro⁷⁰-Gly⁷² in domain 1 of ICAM-1 all result in loss of function blocking mAb epitopes (Berendt et al., 1992), and LFA-1 binding is most severely disrupted by domain 1 mutations Glu³⁴/Ala (on β -strand C) and Gln⁷³/His (on β -strand G) (Staunton et al., 1990). These two residues are conserved in ICAM-2 (Staunton et al., 1989a) and ICAM-3 (Fawcett et al., 1992), as well as in murine ICAM-1 and ICAM-2 (Horley et al., 1989; Xu et al., 1992). Mutation of Asp²⁶-Lys²⁹ or Gly⁴⁶-Asn⁴⁸ or substitution of Lys³⁹, Met⁶⁴ or Tyr⁶⁶ also reduce binding of LFA-1 (Fisher et al., 1997; Staunton et al., 1990). These residues all lie on the apex and the face formed by the C, F and G β -strands of domain 1, indicating that this face of ICAM-1 contains the LFA-1 binding site (see Fig. 3.1).

No specific function has been attributed to domain 2 of ICAM-1 other than to be a spacer to extend domain 1 away from the cell surface. As domain 1 and 2 are structurally inter-dependent, it has not been possible to test either domain for function independently of the other. However, four mAbs that block LFA-1 interaction have been mapped to domain 2 of ICAM-1, suggesting that domain 2 may be involved in LFA-1 binding (Berendt et al., 1992; Duperray et al., 1997; Staunton et al., 1990). The epitopes for these mAbs are located at the top of domain 2. Therefore, rather than their epitopes being sites important for LFA-1 binding, the mAbs may simply be sterically hindering LFA-1 binding to domain 1. For vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the most homologous members of the IgSF to the ICAMs, residues in domain 2 contribute to their binding to the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (Newham et al., 1997). More extensive mutational analysis of domain 2 of ICAM-1 must be carried out before its role is clear.

As for ICAM-1, the two most N-terminal domains of ICAM-3 are sufficient to support LFA-1 binding (Fawcett et al., 1992). ICAM-3 has 47% identity with ICAM-1, with 37% in domain 1 and 77% in domain 2. Although residues Glu³⁴ and Gln⁷³ of ICAM-1, which are critical for binding to LFA-1, are conserved in ICAM-3 (Fawcett et al., 1992), there is evidence that ICAM-1 and ICAM-3 bind to LFA-1 differently. Two mAbs that recognise the LFA-1 I domain inhibit T cell binding to ICAM-3 but not to ICAM-1, and a third I domain mAb activates T cell binding to ICAM-1 but not to ICAM-3 (Landis et al., 1994). In addition, peptide studies indicate that the ICAM-1 and ICAM-3 binding sites in the I domain differ (see Chapter 4.2.9).

As well as binding LFA-1, ICAM-1 acts as a receptor for *Plasmodium falciparum* infected erythrocytes, mediating their adhesion to capillary endothelium, a critical step in the life cycle of the parasite (Berendt et al., 1989). *P. falciparum* causes the most severe form of malaria. Although only a minority of infected

individuals develop life-threatening complications, such as cerebral malaria, it is estimated that as many as 2-3 million deaths each year can be attributed to *P. falciparum* infection. It has been acknowledged for many years that malaria exerts a selective pressure on the human genome, and there is now a growing list of gene polymorphisms that have been recognised to influence the severity of *P. falciparum* infection. Since ICAM-1 is important for the pathogenesis of cerebral malaria, it was considered possible that evolutionary selection by malaria may have resulted in polymorphisms in ICAM-1 that are protective against severe disease. This hypothesis was tested by sequencing the ICAM-1 gene from people living in Kilifi, Kenya, an area of endemic malaria transmission. This study revealed that 14% of the population are homozygous for a single nucleotide substitution that results in the mutation Lys²⁹/Met in domain 1 of ICAM-1 (termed ICAM-1^{Kilifi}). Unexpectedly, however, this polymorphism was found to be associated with increased susceptibility to cerebral malaria (Fernandez-Reyes et al., 1997). Since the polymorphism is found at high frequency despite being linked to such a disadvantageous outcome, it is speculated that it must be maintained by some compensatory selective pressure. The advantage of the Lys²⁹/Met mutation in ICAM-1 to ICAM-1-dependent functions is therefore of interest.

In the past few years I have been involved in a number of collaborations that have had the aim of defining LFA-1 binding sites on the intercellular adhesion molecules ICAM-1 and ICAM-3. My contributions to these studies are described in this chapter. In brief, these pieces of work investigate (1) whether ICAM-1 domain 2 has a role in directly binding to LFA-1, (2) binding sites for LFA-1 in ICAM-3, and (3) the effect of the ICAM-1^{Kilifi} polymorphism on binding of LFA-1.

3.2 RESULTS - THE ROLE OF ICAM-1 DOMAIN 2 IN BINDING LFA-1

3.2.1 Mutations in domain 2 of a three domain ICAM-1Fc construct result in protein which is produced at low levels and which has a severely disrupted structure

Studies I have been involved in previously have shown that four mAbs that recognise epitopes in domain 2 of ICAM-1 are able to inhibit T cell binding to ICAM-1, providing evidence that domain 2 of ICAM-1 has a role in binding to LFA-1 (Berendt et al., 1992; Duperray et al., 1997). It was decided to further investigate this possibility by making mutations in domain 2 of an ICAM-1Fc construct. This project was carried out in collaboration with P. Stanley and J. Brashaw, Leukocyte Adhesion Laboratory, ICRF and with P. Bates, Biomolecular Modelling Laboratory, ICRF.

As hydrophilic, and therefore exposed, residues are likely binding sites, domains 1 and 2 of ICAM-1 were analysed using a Kyte and Doolittle hydrophobicity plot (Kyte and Doolittle, 1982) (data not shown). The CC' and EF loops in domain 2 were found to be the most hydrophilic. Arg¹²⁵GlyGlu, the only residues in the CC' loop, and His¹⁵²HisGly, the most hydrophilic residues in the EF loop, were mutated to alanines (referred to as R¹²⁵GE/AAA ICAM-1Fc and H¹⁵²HG/AAA ICAM-1Fc, respectively). The positions of these loops are indicated in **Figure 3.1**. Preliminary studies using peptides from these loops had indicated that residues on either side of His¹⁵²HisGly might be important in binding LFA-1 (J. Brashaw – unpublished results). Two further mutants were therefore made, R¹⁴⁹RD/AAA and N¹⁵⁶/A. Mutated ICAM-1Fc chimeric proteins were produced by transient transfection of COS-1 cells followed by purification of the secreted protein from the culture supernatant by Protein A Sepharose affinity chromatography.

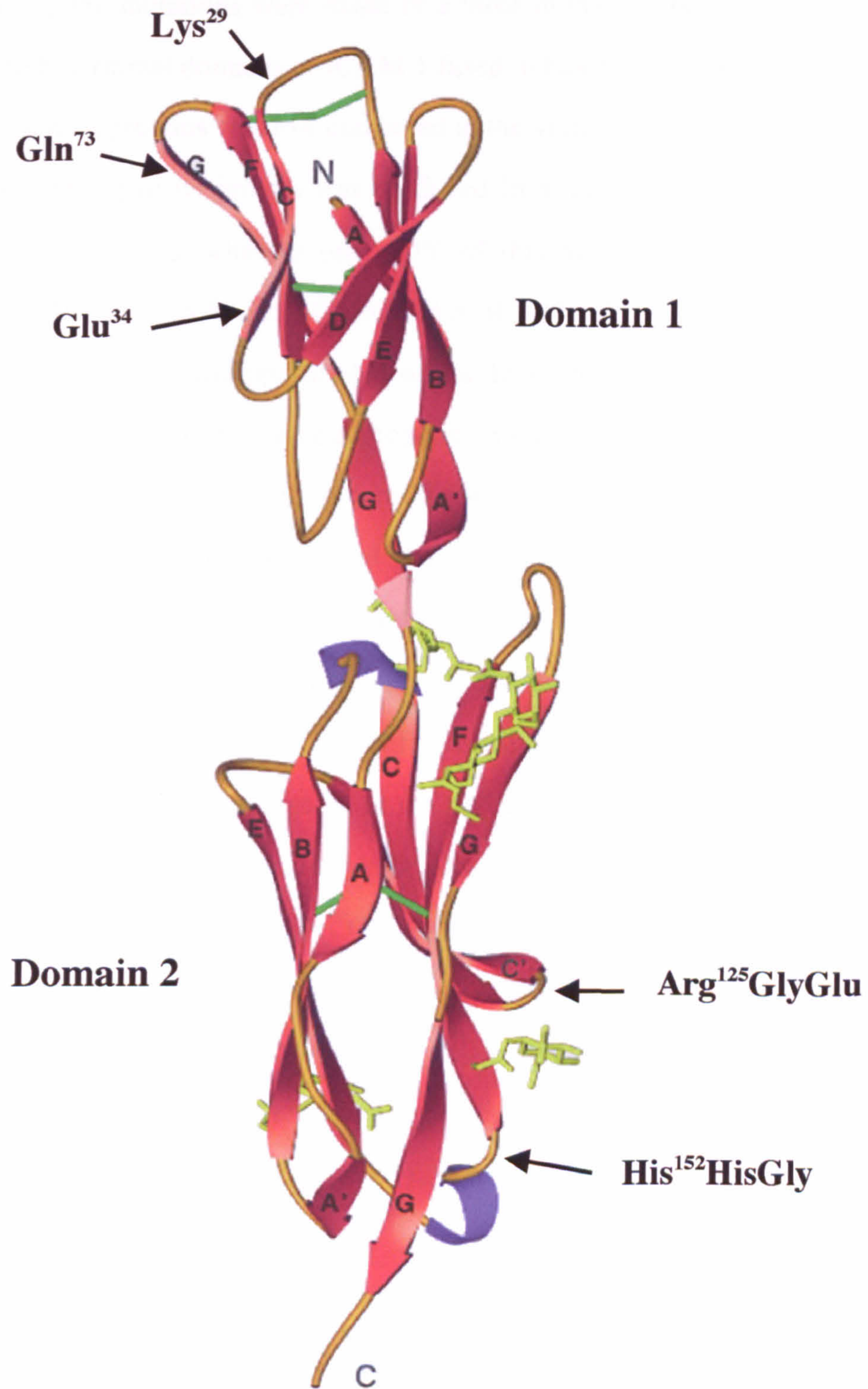


Figure 3.1 Structure of domains 1 and 2 of ICAM-1

In domain 1 Glu³⁴ and Gln⁷³, which are essential for LFA-1 binding (Staunton et al., 1990), are indicated. Lys²⁹ in domain 1 and Arg¹²⁵GlyGlu and His¹⁵²HisGly in domain 2, which are mutated in this study, are also shown. This figure is adapted from Casasnovas et al., 1998b.

Initially the mutations were made in a three domain version of ICAM-1Fc (i.e. the three N-terminal domains of ICAM-1 fused to human IgG₁ Fc). The yield of most of the mutant proteins was low compared to the yield of wild type (wt) protein. Typically 80-100 µg of wt protein was produced from each 15 cm culture dish of transfected COS-1 cells, whereas only 20% of this amount of mutant proteins R¹²⁵GE/AAA, R¹⁴⁹RD/AAA and H¹⁵²HG/AAA ICAM-1Fc were obtained. The N¹⁵⁶/A ICAM-1Fc mutant was produced at similar levels to the wt. It was decided to investigate at what stage of the protein preparation the problem that resulted in low yield of the mutant proteins lay. COS-1 cells transfected with wt ICAM-1Fc cDNA or with cDNA of one of the low yield mutants, R¹²⁵GE/AAA ICAM-1Fc, were labelled with ³⁵S-methionine and the amount of labelled ICAM-1Fc in the cell lysates and secreted into the culture medium compared. Following transfection with both 1 µg and 5 µg of cDNA, wt ICAM-1Fc could be detected as an approximately 80 kDa band both within the cell and secreted in the cell culture supernatant (Fig. 3.2 A lanes 1 and 2). No equivalent band could be detected in mock transfected cells (Fig. 3.2 A lane 5). Very little secreted R¹²⁵GE/AAA ICAM-1Fc could be detected, even when 5 µg of cDNA were transfected (Fig. 3.2 A lanes 3 and 4). The amount of R¹²⁵GE/AAA ICAM-1Fc detected within the cell was also reduced compared to wt (Fig. 3.2 A lanes 3 and 4). Therefore, either there is a defect in synthesis and secretion of R¹²⁵GE/AAA ICAM-1Fc or R¹²⁵GE/AAA ICAM-1Fc is easily degraded, thus resulting in low protein yield.

The small amounts of R¹²⁵GE/AAA, R¹⁴⁹RD/AAA and H¹⁵²HG/AAA ICAM-1Fc protein that were obtained were tested by ELISA for binding of a panel of mAbs that recognise different epitopes in domains 1 and 2 of ICAM-1 (Berendt et al., 1992). When compared to an equal amount of wt ICAM-1Fc, all three mutant proteins showed reduced binding, or lack of binding, of many of the mAbs (Fig. 3.2 B), indicating that the structure of these mutants was severely disrupted. N¹⁵⁶/A ICAM-1Fc, however, which was produced at similar levels to wt, bound all

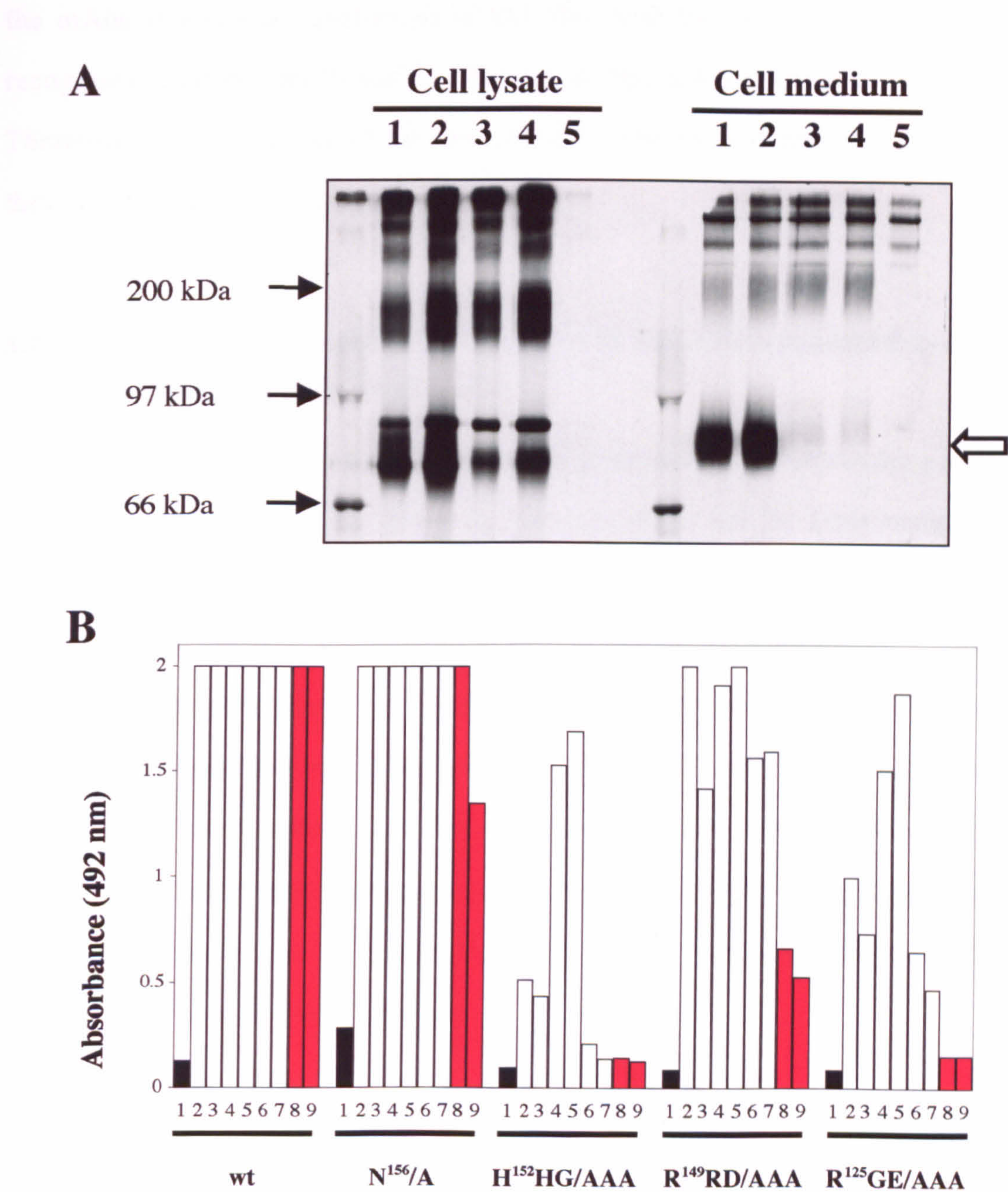


Figure 3.2 Three domain ICAM-1Fc with mutation R¹²⁵GE/AAA is synthesised at reduced level, is poorly secreted, and does not have an intact structure.
A: Protein A Sepharose affinity purified proteins from the cell lysate and cell growth medium of ³⁵S-methionine labelled COS-1 cells transfected with 1 µg wt ICAM-1Fc (lane 1), 5 µg wt ICAM-1Fc (lane 2), 1 µg R¹²⁵GE/AAA ICAM-1Fc (lane 3), 5 µg R¹²⁵GE/AAA ICAM-1Fc (lane 4), or mock transfected (lane 5). The open arrow indicates the expected position of the ICAM-1Fc proteins. **B:** An ELISA to test the binding of a panel of ICAM-1 mAbs (used as neat supernatant, 1/100 ascites or 10 µg/ml purified mAb) to wt and mutated ICAM-1Fc (all at 10 µg/ml). The mAbs used are 38 (1), My13 (2), 7F7 (3), RR1/1 (4), WEHI-CAM-1 (5), 7.5C2 (6), 15.2 (7), 8.4A6 (8) and R6.5 (9). These mAbs recognise domain 1 (□) or domain 2 (■) of ICAM-1 except the negative control mAb (■) which recognises CD11a. Data are from representative experiments (n=2).

the mAbs at a similar level to wt ICAM-1Fc, with the exception of R6.5, which recognises residues Glu¹¹¹-Ala¹¹⁴ at the top of domain 2 (Staunton et al., 1990). Therefore, as the majority of the three domain constructs appeared to be unstable, they could not be used for further studies.

3.2.2 A five domain version of R¹²⁵GE/AAA ICAM-1Fc is produced as a secreted protein by COS-1 cells

Attempts to improve the protein yield were made to disprove the possibility that, rather than the mutations disrupting the structure, it was the extra manipulations required for production of low yield proteins, for example concentration, which were responsible for the loss of structure. Two approaches were tested: electroporation was tried as an alternative to DEAE dextran to facilitate transfection of COS-1 cells; and the mutant proteins were made as five domain constructs (i.e. the five N-terminal domain of ICAM-1 fused to human IgG₁ Fc). Electroporation did not prove any better than DEAE dextran as again very little R¹²⁵GE/AAA ICAM-1Fc was secreted (Fig. 3.3 A lane 3), but N¹⁵⁶/A ICAM-1Fc was secreted into the medium at similar levels to wt (Fig. 3.3 A lane 1 and 2).

The five domain version of the ICAM-1Fc construct has a different Fc portion to the three domain construct and is able to dimerise, thus the five domain proteins run on SDS-PAGE under non-reducing conditions as an approximately 200 kDa band. ³⁵S-methionine labelling of COS-1 cells transfected with cDNA for five domain R¹²⁵GE/AAA ICAM-1Fc showed that this form of the protein could be secreted (Fig 3.3 B lane 2), whereas, as seen previously, two different cDNA clones of the three domain version were not secreted (Fig. 3.3 B lanes 3 and 4). The five domain constructs were therefore used in all further experiments.

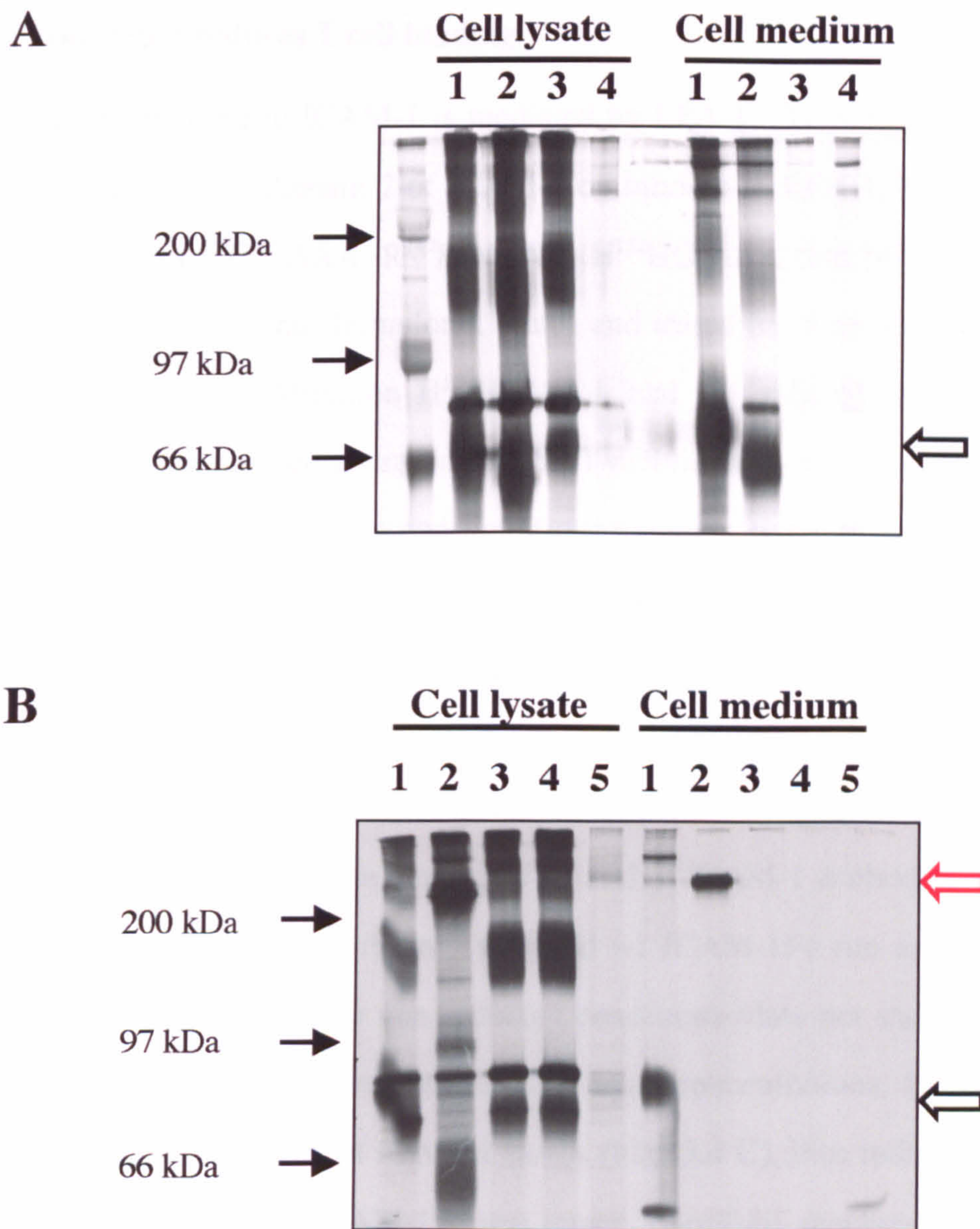


Figure 3.3 Three domain ICAM-1Fc with mutation R¹²⁵GE/AAA is synthesised at reduced level and poorly secreted from electroporated cells, however a five domain version is secreted.

Protein A Sepharose affinity purified proteins from the cell lysate and cell growth medium of ³⁵S-methionine labelled COS-1 cells transfected **A:** by electroporation with three domain versions of wt ICAM-1Fc (lane 1), N¹⁵⁶/A ICAM-1Fc (lane 2), R¹²⁵GE/AAA ICAM-1Fc (lane 3), or mock transfected (lane 4) or **B:** by DEAE dextran with three domain wt ICAM-1Fc (lane 1), five domain R¹²⁵GE/AAA ICAM-1Fc (lane 2), three domain R¹²⁵GE/AAA ICAM-1Fc clone 1 (lane 3), three domain R¹²⁵GE/AAA ICAM-1Fc clone 2 (lane 4), or mock transfected (lane 5). The expected positions of the 3 domain and five domain ICAM-1Fc proteins are indicated by the black open arrows and the red open arrow respectively.

3.2.3 Mutation H¹⁵²HG/AAA in domain 2 of a five domain ICAM-1Fc construct reduces T cell binding

T cell binding to ICAM-1 is mediated by LFA-1. Therefore, to assess the effect of mutations in domain 2 of ICAM-1 on binding to LFA-1, the five domain versions of wt, R¹²⁵GE/AAA, R¹⁴⁹RD/AAA, H¹⁵²HG/AAA and N¹⁵⁶/A ICAM-1Fc proteins were titrated onto Immulon-1 plates and tested for their ability to support adhesion of T cells. Mutation H¹⁵²HG/AAA had the greatest effect on T cell adhesion, showing reduced ability to support T cell binding compared to wt ICAM-1Fc (Fig. 3.4 A). At saturating concentrations the other mutants supported similar levels of T cell adhesion to wt ICAM-1Fc, although binding to all the mutants was reduced to some extent at sub-saturating concentrations (Fig. 3.4 B). To ensure that these results were due to the H¹⁵²HG/AAA mutation affecting LFA-1 binding and not due to a gross defect in the structure of the molecule, the integrity of the protein was checked by SDS-PAGE and the binding of a panel of ICAM-1 antibodies was tested. SDS-PAGE showed that all the mutants and wt ICAM-1Fc run as one band of approximately 200 kDa under non-reducing conditions (data not shown). Wt and H¹⁵²HG/AAA ICAM-1Fc, when compared at equal concentrations, showed similar levels of binding of a panel of ICAM-1 mAbs (Fig. 3.4 C), thus indicating that the structure of H¹⁵²HG/AAA ICAM-1Fc was intact. Therefore, residues His¹⁵²HisGly, which lie in the EF loop at the bottom of domain 2 of ICAM-1, have a role in ICAM-1 binding to LFA-1.

When the T cell binding assays were performed with the ICAM-1Fc proteins captured on plates coated with an antibody against the Fc portion of human IgG, the same pattern of results were seen as when the ICAM-1Fc proteins were coated directly on plastic (data not shown). These results dismiss the possibility that the reduced T cell binding to HHG/AAA ICAM-1Fc was due to lower coating density on the plates.

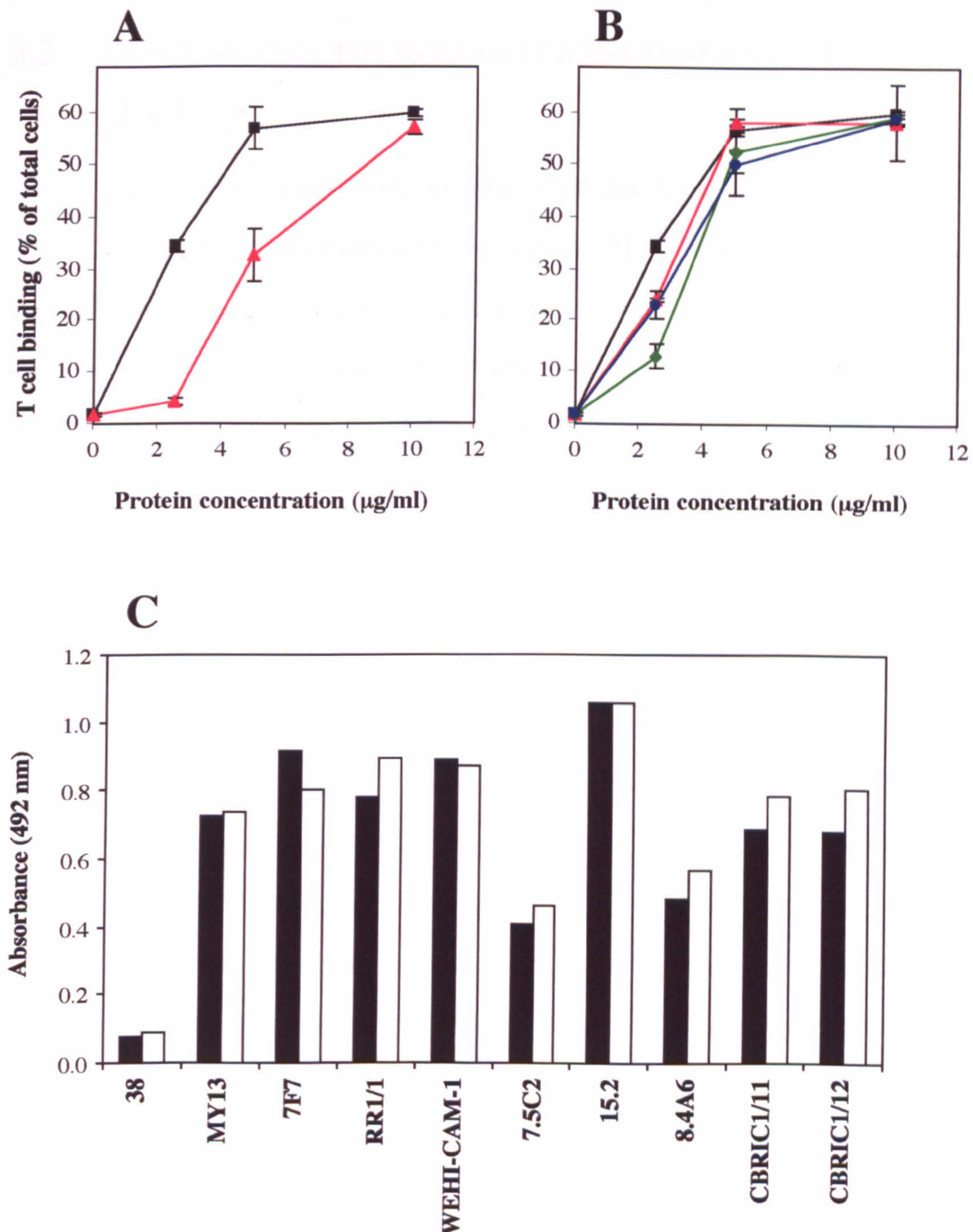


Figure 3.4 Mutation H¹⁵²HG/AAA in ICAM-1Fc disrupts T cell binding.

T cell binding stimulated by 2 mM MgCl₂/1 mM EGTA to 0-10 μg/ml **A:** wt ICAM-1Fc (■) and H¹⁵²HG/AAA ICAM-1Fc (▲) and **B:** wt ICAM-1Fc (■), R¹²⁵GE/AAA ICAM-1Fc (▲), R¹⁴⁹RD/AAA ICAM-1Fc (◆) and N¹⁵⁶/A ICAM-1Fc (●). Data (mean of triplicates ± S.D.) from one representative experiment (n=4) are shown. **C:** An ELISA to test the binding of a panel of ICAM-1 mAbs (used as neat supernatant, 1/100 ascites or 10 μg/ml purified mAb) to 10 μg/ml wt ICAM-1Fc (■) and H¹⁵²HG/AAA ICAM-1Fc (□). Data from one representative experiment (n=3) are shown.

3.3 DISCUSSION - THE ROLE OF ICAM-1 DOMAIN 2 IN BINDING LFA-1

The first part of this work has established that domain 2 of ICAM-1 has a role in binding to LFA-1. In particular, the residues His¹⁵²HisGly, which lie in the EF loop at the bottom of domain 2, prove to be important. It was not clear from this work, however, whether residues His¹⁵²HisGly directly bind LFA-1, or whether their mutation indirectly affects the LFA-1 binding site in domain 1. These possibilities were investigated by P. Stanley in the laboratory (Stanley et al., 2000). She showed that mutation of His¹⁵²HisGly prevented binding to the isolated I domain of LFA-1. Mutation of Glu³⁴ in domain 1 of ICAM-1 has already been shown to prevent binding to the I domain (Stanley and Hogg, 1998), in keeping with the hypothesis that Glu³⁴ coordinates the divalent cation bound to the I domain (Lee et al., 1995b). Although the two N-terminal domains of ICAM-1 contain a similar number of residues as the I domain, the I domain is a much more compact structure, being approximately 25 Å in diameter compared to the 38 Å diameter of domain 1 of ICAM-1. The size of the I domain relative to an Ig-like domain makes it impossible for the I domain to bind to Glu³⁴ in domain 1 and to His¹⁵²HisGly in domain 2 at the same time. Further evidence against His¹⁵²HisGly directly binding to the I domain comes from the crystal structure of ICAM-1, which reveals that His¹⁵²HisGly and Glu³⁴ are on opposite sides of the molecule (see Fig. 3.1) (Casasnovas et al., 1998b). In support of an indirect effect by His¹⁵²HisGly on I domain binding, the I domain was unable to bind to a chimeric protein in which domain 1 of ICAM-1 was replaced with domain 1 of VCAM-1. Using this construct it was also shown that the ICAM-1 domain 2 mAbs, which had been shown to inhibit interaction with LFA-1, do this indirectly (Stanley et al., 2000). Therefore domain 2 of ICAM-1 appears to have a critical role in binding to LFA-1, but does not bind directly.

It was unexpected that a three domain ICAM-1Fc construct was misfolded and poorly synthesised upon mutation of some residues in domain 2, but that these problems were not encountered when the same mutations were made in a five domain ICAM-1Fc construct. The five domain constructs differ from the three domain constructs in that they have a Fc portion that allows dimerisation. Whether dimerisation or the presence of all five domains contributes to the stability of the protein is unclear. Proper folding of domain 1 of ICAM-1 is dependent on domain 2 (Stanley et al., 2000; Staunton et al., 1990), therefore it is possible that mutation of domain 2 could affect the structure of domain 1. Another study has found that folding at the bottom of domain 2 affects binding of rhinovirus to domain 1 (Casasnovas et al., 1998a), providing evidence that alterations in structure in one domain can be transmitted through the molecule to affect the structure of another domain.

3.4 RESULTS - BINDING SITES IN ICAM-3 FOR LFA-1

3.4.1 Identification of ICAM-3 mAbs that inhibit interaction with LFA-1

As domain 1 and 2 of ICAM-3 had been shown to contain the LFA-1 binding site (Fawcett et al., 1992), it was decided to further localise the binding site by a combination of mutagenesis of these two domains and mapping of function blocking mAbs. This work was done in collaboration with C. Holness and D. Simmons, Cell Adhesion Laboratory, ICRF, Oxford and P. Bates Biomolecular Modelling Laboratory, ICRF.

First, to identify function-blocking mAbs, a panel of 16 ICAM-3 mAbs, obtained from Leukocyte Typing V, was tested for ability to block T cell binding to ICAM-3Fc immobilised on Immulon-1 plates. Although this interaction was completely inhibited by LFA-1 mAb 38, all the ICAM-3 mAbs (at 10 µg/ml or 1/100 dilution of ascites) failed to affect T cell adhesion (**Fig. 3.5**). Four ICAM-3 mAbs that were not submitted to the Leukocyte Typing Workshop, CH3.1, CH3.2, CH3.3 and KS118, were tested separately and showed no effect on T cell binding to ICAM-3 (data not shown). These four mAbs were only available in the form of tissue culture supernatant and, therefore, may not have been tested at optimum concentration. In conclusion, these experiments failed to identify any function-blocking ICAM-3 mAbs.

3.4.2 Structural integrity of ICAM-3Fc mutants and mapping of ICAM-3 mAbs

Based on the results of ICAM-1 mutagenesis, residues predicted to be exposed on the CFG face of ICAM-3 domain 1 were substituted in an ICAM-3Fc construct containing domains 1 and 2 of ICAM-3 (see **Fig. 3.6**). Connecting loops between β -strands in domain 2 were also mutated (see **Fig. 3.6**). The ICAM-3 mAbs

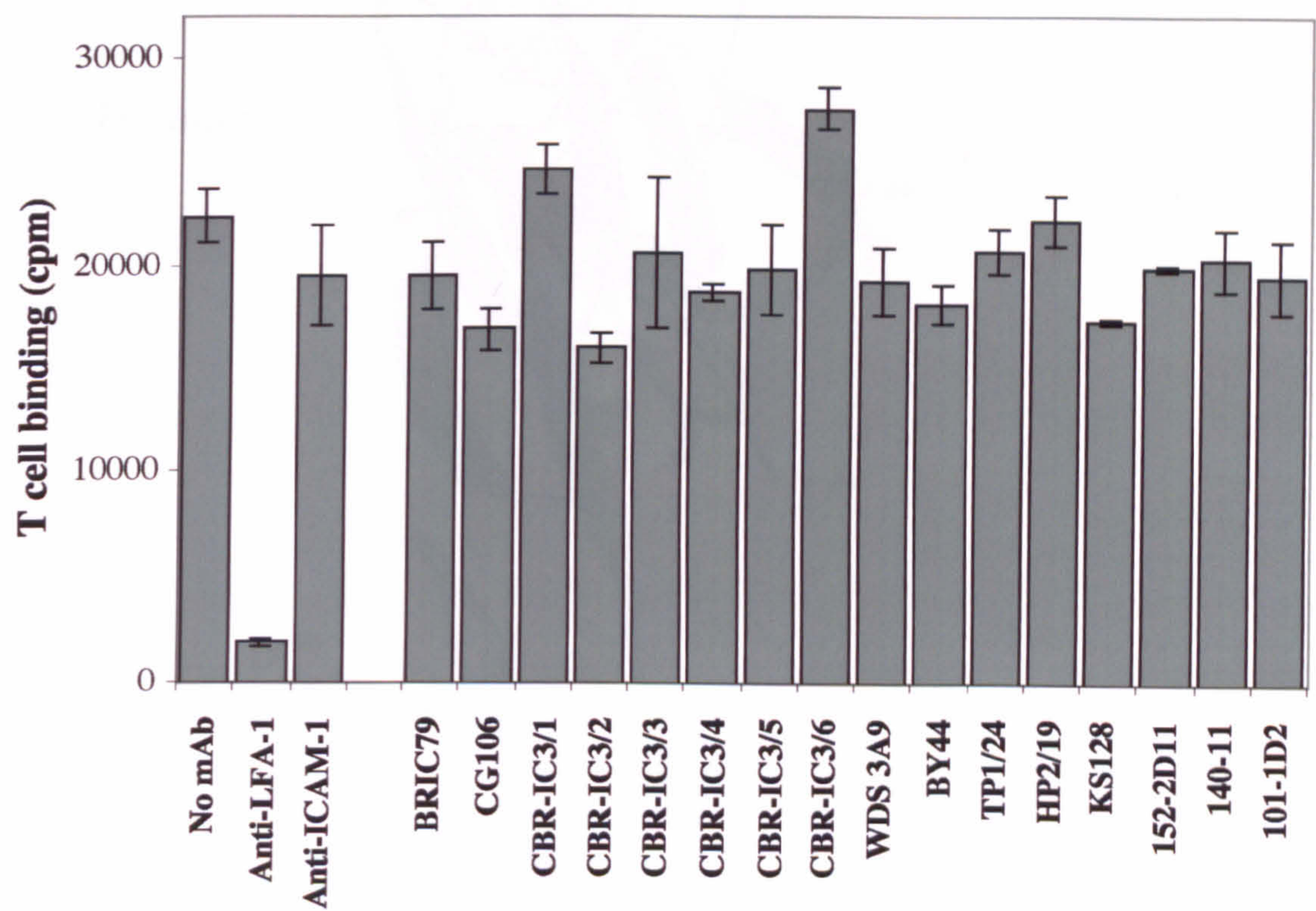


Figure 3.5 Effect of ICAM-3 mAbs on T cell binding to ICAM-3
T cell binding to ICAM-3Fc stimulated by 2 mM MgCl₂/1 mM EGTA/50 nM PdBu in the presence of the indicated mAbs (1/100 dilution). Data (mean of triplicates ± S.D.) from one representative experiment (n=3) are shown.

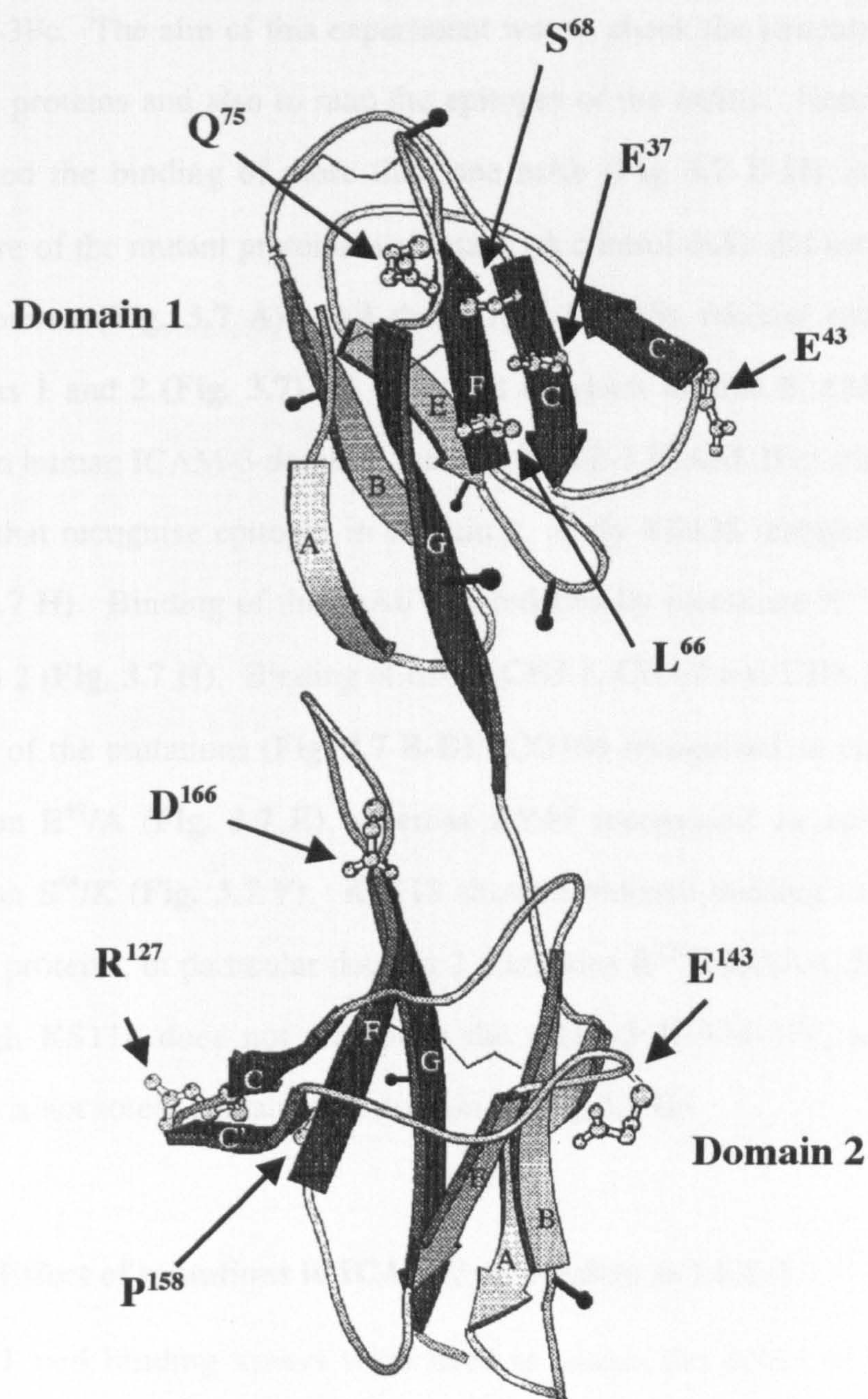


Figure 3.6 A model of domains 1 and 2 of ICAM-3

This model of domains 1 and 2 of ICAM-3 was produced by P. Bates, Biomolecular Modelling Laboratory, ICRF. The locations of residues subjected to site-directed mutagenesis are shown.

that were in most abundant supply were tested for reactivity with wt and mutated ICAM-3Fc. The aim of this experiment was to check the structural integrity of the mutant proteins and also to map the epitopes of the mAbs. None of the mutations disrupted the binding of more than one mAb (Fig 3.7 B-H), indicating that the structure of the mutant proteins was intact. A control mAb did not react with any of the proteins (Fig. 3.7 A). All the ICAM-3 mAbs reacted with wt ICAM-3Fc domains 1 and 2 (Fig. 3.7). A construct in which murine ICAM-1 domain 1 was fused to human ICAM-3 domains 2 and 3 (m1h2-3 ICAM-3Fc) was used to identify mAbs that recognise epitopes in domain 2. Only KS128 recognised this construct (Fig. 3.7 H). Binding of this mAb was reduced by mutations R¹²⁷/A and D¹⁶⁶/A in domain 2 (Fig. 3.7 H). Binding of mAbs CH3.1, CH3.2 and CH3.3 was not affected by any of the mutations (Fig. 3.7 B-D). CG106 recognised an epitope affected by mutation E³⁷/A (Fig. 3.7 E), whereas BY44 recognised an epitope affected by mutation S⁶⁸/K (Fig. 3.7 F). KS118 showed reduced binding to a number of the mutant proteins, in particular domain 2 mutations R¹²⁷WE/AAA, R¹²⁷/A and D¹⁶⁶/A, although KS118 does not recognise the m1h2-3 ICAM-3Fc, indicating that its epitope is not solely contained in domain 2 (Fig. 3.7 G).

3.4.3 Effect of mutations in ICAM-3 on binding to LFA-1

T cell binding assays were used to assess the effect of the mutations in ICAM-3 on LFA-1 binding and to determine whether the ICAM-3 binding site for LFA-1 is similar to the ICAM-1 site. T cell binding was totally abolished by mutations E³⁷/A, L⁶⁶/K, S⁶⁸/K, Q⁷⁵/A and Q⁷⁵/H (Fig. 3.8 A and B). The domain 2 mutations E¹⁴³/A, P¹⁵⁸/A and D¹⁶⁶/A had little effect. T cell binding to domain 1 mutant E⁴³/A and the domain 2 mutants R¹²⁷/A and R¹²⁷WE/AAA was consistently lower than to wt.

Similar results were obtained when the ICAM-3Fc proteins were captured on plates coated with an antibody against the Fc portion of human IgG (data not shown).

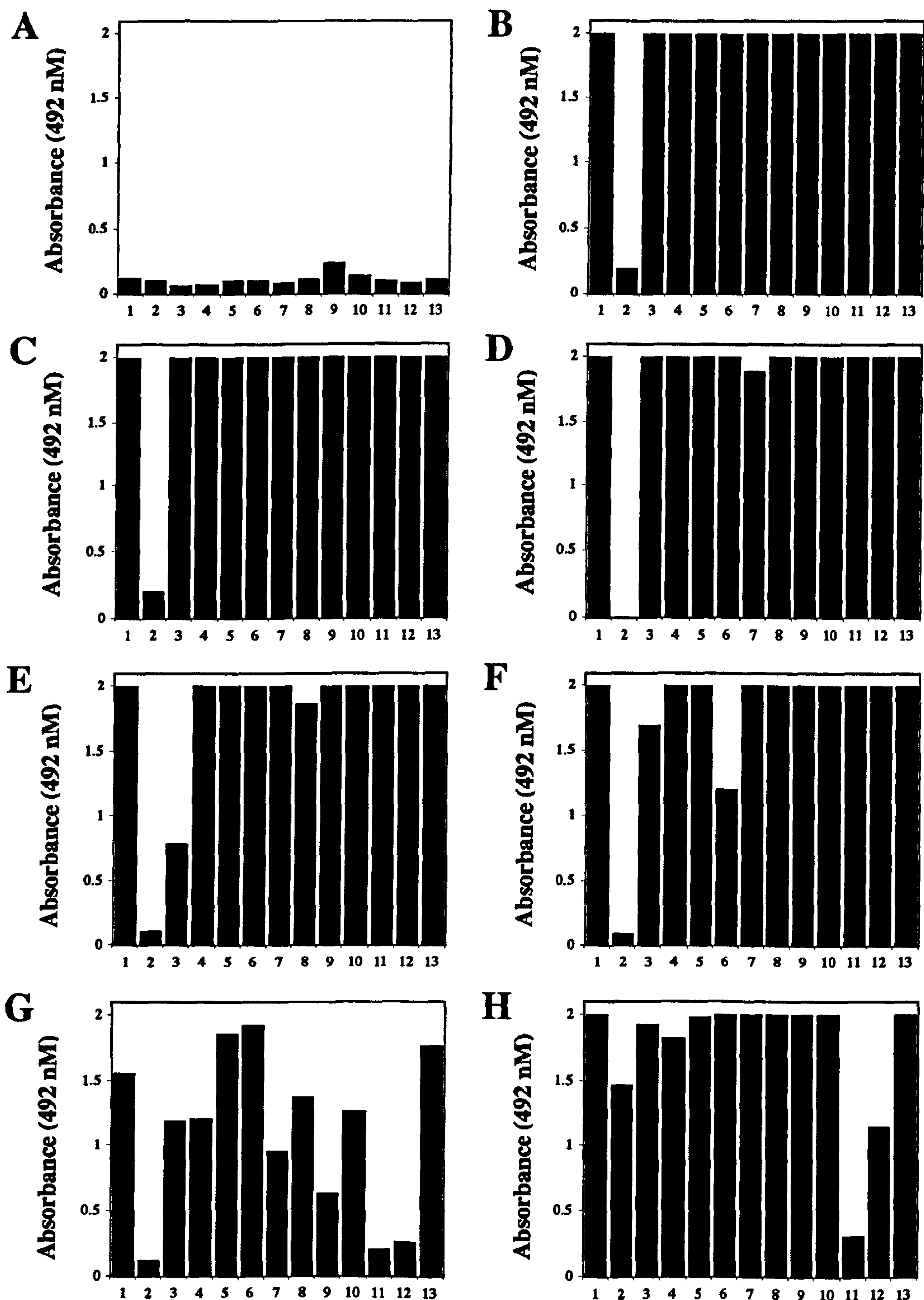


Figure 3.7 Mapping of ICAM-3 mAbs

An ELISA to test the binding of a panel of ICAM-3 mAbs to wt ICAM-3Fc (1), m1h2-3 ICAM-3Fc (2), or ICAM-3Fc with mutation E³⁷/A (3), E⁴³/A (4), L⁶⁶/K (5), S⁶⁸/K (6), Q⁷⁵/A (7), Q⁷⁵/H (8), R¹²⁷WE/AAA (9), E¹⁴³/A (10), D¹⁶⁶/A (11), R¹²⁷/A (12), P¹⁵⁸/A (13). A: ICAM-1 mAb 15.2 was used as a negative control. The ICAM-3 mAbs used are B: CH3.1, C: CH3.2, D: CH3.3, E: CG106, F: BY44, G: KS118, and H: KS128. All proteins were tested at 10 µg/ml and all mAbs were used as neat supernatant. Data from one representative experiment (n=3) are shown.

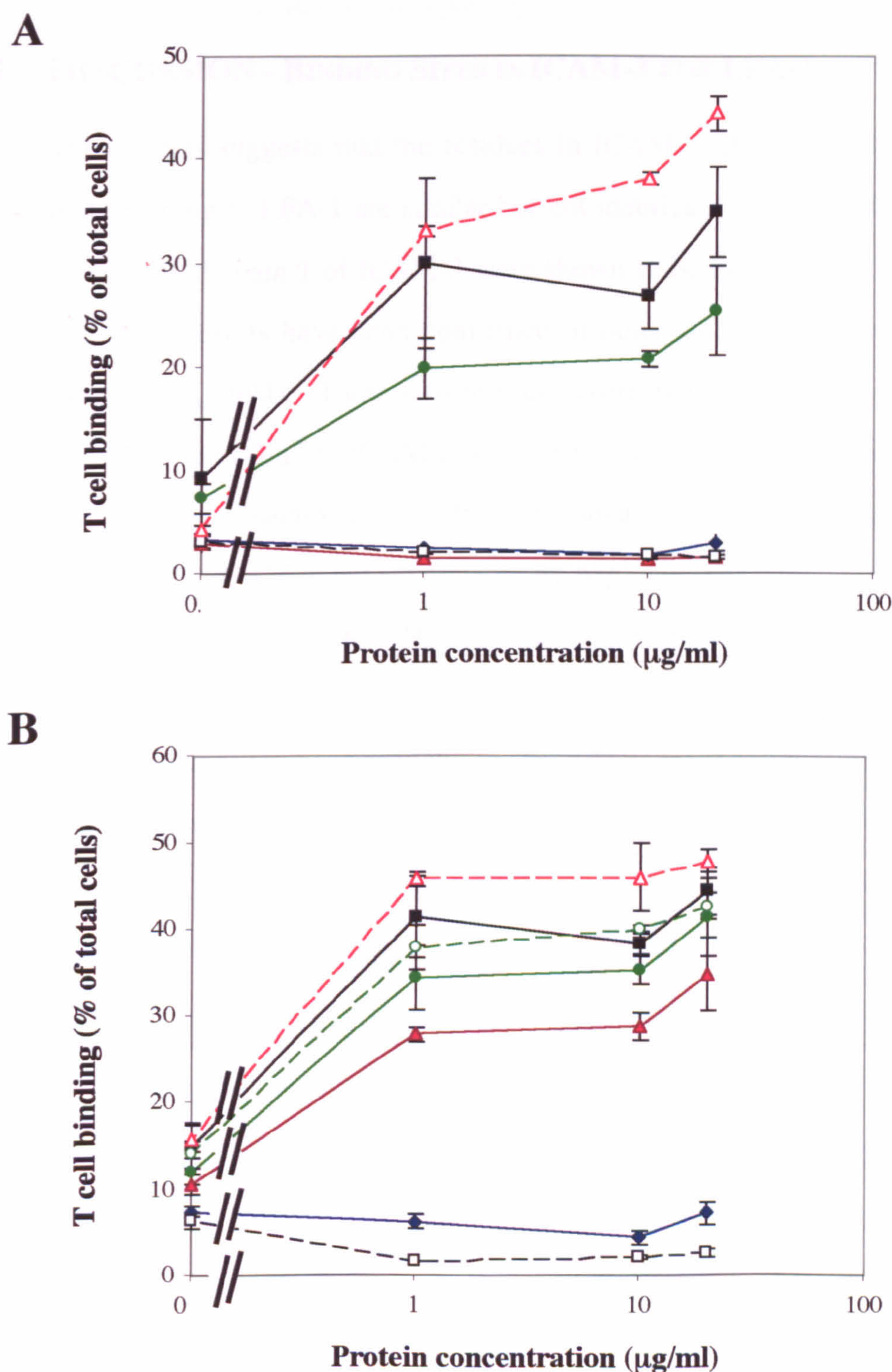


Figure 3.8 Effect of mutations in ICAM-3 domains 1 and 2 on T cell binding

2 mM MgCl_2 /1 mM EGTA/50 nM PdBu stimulated T cell binding to 0-20 $\mu\text{g/ml}$ **A:** wt ICAM-3Fc (■) or ICAM-3Fc with mutation E^{37}/A (▲), E^{43}/A (●), Q^{75}/A (◆), Q^{75}/H (□), P^{158}/A (△), and **B:** wt ICAM-3Fc (■), or ICAM-3Fc with mutation $\text{R}^{127}\text{WE}/\text{AAA}$ (▲), R^{127}/A (●), L^{66}/K (◆), S^{68}/K (□), E^{143}/A (△), D^{166}/A (○). Data (mean of triplicates \pm S.D.) from one representative experiment ($n=5$) are shown.

3.5 DISCUSSION - BINDING SITES IN ICAM-3 FOR LFA-1

Mutagenesis suggests that the residues in ICAM-1, ICAM-2 and ICAM-3 that mediate binding to LFA-1 are similar but not identical. In this study residues Glu³⁷ and Gln⁷⁵ in domain 1 of ICAM-3 were shown to be essential for binding to LFA-1 and these results have been confirmed in other studies (Klickstein et al., 1996; Sadhu et al., 1994). These two residues correspond to Glu³⁴ and Gln⁷³ of ICAM-1 and Glu³⁷ and Gln⁷⁵ of ICAM-2, which have also been shown to be essential for binding to LFA-1 (Staunton et al., 1990; Casasnovas et al., 1999). These residues are not conserved in ICAM-4, but are replaced by Arg⁵² and Thr⁹¹, mutation of which has no effect on binding to LFA-1 (Hermant et al., 2000). In ICAM-5, Glu⁴⁰ is the equivalent of Glu³⁷, but Thr⁷⁹ occupies the equivalent of the second position. However, information is not yet available on the role of these residues in binding to LFA-1. Mutation of Glu³⁴ in ICAM-1 prevents binding to a recombinant form of the LFA-1 I domain (Stanley and Hogg, 1998), and it is proposed that this residue coordinates Mg²⁺ bound to the I domain (Lee et al., 1995b).

The crystal structure of ICAM-3 is not yet available but it is predicted that, as for ICAM-1 and ICAM-2, Glu³⁷ lies in strand C and Gln⁷⁵ in strand G. Other residues on the CFG face were shown to be important for binding to LFA-1, namely Leu⁶⁶ and Ser⁶⁸. In other studies Glu³², Lys³³, Thr³⁸, Lys⁴², Phe⁵⁴ and Arg⁶⁴ of ICAM-3 have all been shown to play a role in the binding site for LFA-1 (Bell et al., 1998; Klickstein et al., 1996; Sadhu et al., 1994). Four positions, corresponding to Glu³⁷, Phe⁵⁴, Ser⁶⁸ and Gln⁷⁵ in ICAM-3, are also important in ICAM-1 and 2 (Casasnovas et al., 1999). Binding to the Lys⁴² ICAM-3 mutant was consistently reduced compared to binding to wt, but was not severely disrupted. The corresponding residue in ICAM-1 and ICAM-2 is essential for binding (Casasnovas et al., 1999; Fisher et al., 1997), and for ICAM-3 it has been shown to be necessary for binding when activation is not optimum (Holness et al., 1995). It has been

suggested that the role of this residue in ICAM-2 may be to orientate the side chain of the critical residue Glu³⁷ (Casasnovas et al., 1999). For ICAM-1 and ICAM-2, the LFA-1 binding site is rectangular in shape and is present on a long flat ridge on domain 1. In contrast to the LFA-1 binding sites on ICAM-1 and ICAM-2, there is evidence that the LFA-1 binding site on ICAM-3 may also include residues on the BED face (Bell et al., 1998; Klickstein et al., 1996).

Unlike for ICAM-1, domain 1 of ICAM-3 can be expressed independently of domain 2 (Klickstein et al., 1996). Experiments with domain deletion mutants of ICAM-3 indicate that domain 1 is sufficient for binding LFA-1 and that domains 2 and 3 do not contribute to the binding site (Klickstein et al., 1996). Others, however, report that mutations in domain 2 of ICAM-3 can diminish LFA-1 binding, including His¹⁵⁵ and Gly¹⁵⁶, which correspond to His¹⁵³ and Gly¹⁵⁴ in ICAM-1 (Bell et al., 1998; Holness et al., 1995). Whether the role of these residues is indirect, as has been shown here for ICAM-1, was not investigated. None of the mutations made in domain 2 were found to have a severe effect on LFA-1 binding. Mutation of R¹²⁷ and R¹²⁷WE in the CC' loop of domain 2 did reduce T cell binding, and in another study, using LFA-1 transfectants, it was shown that the effect of these mutations is more pronounced when activation of LFA-1 is not optimum (Holness et al., 1995). The equivalent residues in ICAM-1 are R¹²⁵GE, which are shown here not to play a role in LFA-1 binding (see Fig. 3.4).

The ICAM-3 mAb CG106 was mapped to an epitope including Glu³⁷, and binding of BY44 was diminished by mutation of Ser⁶⁸. These two mutations completely prevented LFA-1 binding, therefore it was surprising to find that CG106 and BY44 did not inhibit T cell binding to ICAM-3. Another study has confirmed the mapping of CG106 to Glu³⁷ and shown that the BY44 epitope also includes Gln⁷⁵. They find, however, that these two mAbs, as well as several others in the Leukocyte Typing V panel, are inhibitory for LFA-1 binding to ICAM-3 (Klickstein et al., 1996). The discrepancy in results remains unexplained but different assays were

used and the mAbs should have been titrated. KS128 maps to domain 2, however it has been reported that its epitope also encompasses residues in domain 1 (Klickstein et al., 1996).

3.6 RESULTS - LFA-1 BINDING TO ICAM-1 WITH THE ICAM-1^{KILIFI} POLYMORPHISM

3.6.1 Effect of the ICAM-1^{Kilifi} polymorphism on structural integrity of the protein

In collaboration with A. Craig, Oxford and D. Altieri, Yale University, USA, the mechanism by which the ICAM-1^{Kilifi} polymorphism is maintained, despite being associated with susceptibility to cerebral malaria, was investigated. The mutation K²⁹/M was introduced into a five domain version ICAM-1Fc (K²⁹/M ICAM-1Fc). Before using this protein in functional assays it was important to ensure that the structure was intact. ELISAs were performed to compare the binding of a panel of ICAM-1 mAbs to equal amounts of wt ICAM-1Fc and K²⁹/M ICAM-1Fc. The results demonstrate that the epitopes for all the mAbs, with the exception of BBA4, are present on K²⁹/M ICAM-1Fc at the same level as on wt ICAM-1Fc (Fig. 3.9 A). Binding of BBA4 has previously been shown to be destroyed by the K²⁹/M mutation (Fernandez-Reyes et al., 1997).

3.6.2 Effect of the ICAM-1^{Kilifi} polymorphism on T cell binding

Having verified the structural integrity of the K²⁹/M ICAM-1Fc protein, it was tested for ability to support LFA-1-mediated T cell adhesion. LFA-1 binding to ICAM-1 is critical for many functions in the immune system, therefore alteration of this interaction could have immunomodulatory effects that provide a compensatory selective advantage for individuals with the ICAM-1^{Kilifi} gene. At protein concentrations that supported maximal T cell binding, similar numbers of cells

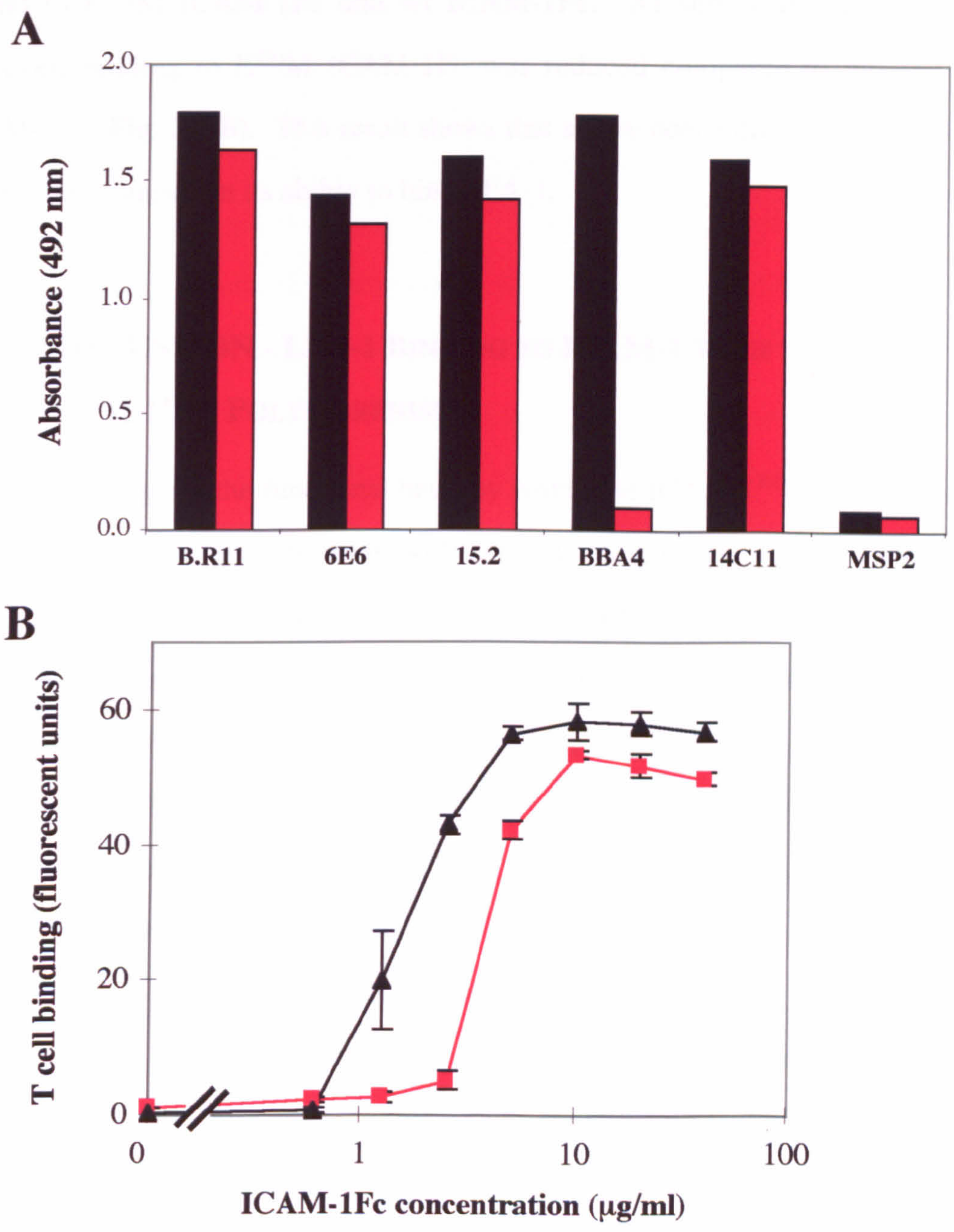


Figure 3.9 Effect of ICAM-1^{Kilifi} polymorphism on structural integrity and T cell binding

A: An ELISA to test the binding of a panel of ICAM-1 mAbs (at 5 $\mu\text{g/ml}$) to 10 $\mu\text{g/ml}$ ICAM-1Fc (■) and ICAM-1^{Kilifi} (■). MSP2 is used as a negative control mAb. Data are from a representative experiment (n=4). **B:** T cell binding to 0-40 $\mu\text{g/ml}$ ICAM-1Fc (▲) and ICAM-1^{Kilifi} (■) stimulated by 50 nM PdBu. Data (mean of triplicates \pm S.D.) are from a representative experiment (n=3).

bound to K²⁹/M ICAM-1Fc and wt ICAM-1Fc. At sub-saturating conditions, however, binding to K²⁹/M ICAM-1Fc was reduced compared to binding to wt ICAM-1Fc (Fig. 3.9 B). This result shows that at low concentrations K²⁹/M ICAM-1Fc is compromised in its ability to bind LFA-1.

3.7 DISCUSSION - LFA-1 BINDING TO ICAM-1 WITH THE ICAM-1^{KILIFI} POLYMORPHISM

To recognise the functional basis by which the ICAM-1^{Kilifi} polymorphism is maintained despite an association with cerebral malaria, an ICAM-1Fc construct with the mutation K²⁹/M was made and compared with wt ICAM-1Fc for the ability to support a number of ICAM-1 functions. As demonstrated in this chapter, K²⁹/M ICAM-1Fc has reduced affinity for LFA-1. LFA-1 binding to ICAM-1 plays a crucial role in the immune system, mediating leukocyte-leukocyte and leukocyte-endothelial interactions. The interaction of ICAM-1 with fibrinogen also mediates leukocyte-endothelial interaction, with fibrinogen acting as a bridge between ICAM-1 on the endothelium and Mac-1 on the leukocyte (Languino et al., 1993). D. Altieri and colleagues found that the binding of K²⁹/M ICAM-1Fc to fibrinogen was severely impaired (Craig et al., 2000). Thus, by reducing leukocyte adhesion, the polymorphism could have beneficial effects, for instance by limiting the leukocyte mediated tissue damage that occurs in response to infection.

Rhinovirus is likely to have an impact on childhood mortality in areas of malarial transmission, if not directly then through secondary bacterial infection. The adherence of rhinovirus to K²⁹/M ICAM-1Fc is one crucial ICAM-1 function that we were unable to investigate in this study. Lys²⁹ lies at the end of the B to C loop in domain 1 of ICAM-1 (see Fig. 3.1). Mutation of residues Asp²⁶-Lys²⁹ in this loop has been shown to reduce rhinovirus binding to 13% of the level of binding to wt

ICAM-1 (Staunton et al., 1990), suggesting that the ICAM-1^{Kilifi} polymorphism could be protective against rhinovirus infection.

Susceptibility to malaria is determined by a complex evolutionary interplay between host and parasite genetics. Therefore, as well as human gene polymorphisms, variants of *P. falciparum* also occur. In previous studies, mutation of Lys²⁹ had no effect on infected erythrocyte binding to ICAM-1 (Berendt et al., 1992; Ockenhouse et al., 1992). However, when two different laboratory isolates of *P. falciparum* were tested for adherence to K²⁹/M ICAM-1Fc it was found by A. Craig and colleagues that binding of one was disrupted but the other not (Craig et al., 2000). These results are likely to reflect a variation in the parasite protein that mediates adhesion to ICAM-1. Geographical variation in *P. falciparum* populations may explain why the ICAM-1^{Kilifi} polymorphism predisposes to cerebral malaria in Kenya (Fernandez-Reyes et al., 1997), but shows no association in The Gambia, an area of low malaria transmission (Bellamy et al., 1998).

3.8 CONCLUSION

In conclusion, the work in this chapter demonstrates that for ICAM-3 similar residues to ICAM-1 and ICAM-2 are involved in binding LFA-1. The main LFA-1 binding site lies on the face of domain 1 of the ICAMs formed by the C, F and G β -strands and the CD edge. Evidence is also provided that, in addition to domain 1, domain 2 of both ICAM-1 and ICAM-3 plays a role in binding LFA-1. Finally, it is shown that the ICAM-1^{Kilifi} polymorphism reduces the affinity of ICAM-1 for LFA-1 and we speculate that this may have an immuno-modulatory effect that could provide an evolutionary selective advantage.

CHAPTER 4

A ROLE FOR THE LFA-1 I DOMAIN IN HIGH AFFINITY BINDING TO ICAM-1

4.1 INTRODUCTION

In this laboratory, two methods have been used to induce LFA-1 on T cells to bind to its ligands. The characteristics of the active LFA-1, however, differ depending on the method of stimulation. The first method is PKC activation by the phorbol ester PdBu, which is thought to mimic signalling following engagement of the T cell receptor or chemokine receptors. PdBu does not induce a detectable increase in LFA-1 affinity, but enhances the avidity of adhesion through a pathway dependent on PKC, intracellular Ca^{2+} and the actin cytoskeleton (Stewart et al., 1996). Further details of this form of adhesion are investigated in **Chapter 5**. The second method is addition of the divalent cation Mg^{2+} with the simultaneous chelation of Ca^{2+} using EGTA. It is thought that Mg^{2+} binds to the MIDAS motif in the I domain and induces a change in the conformation of LFA-1 that allows ligand binding. Activation with Mg^{2+} increases the affinity of LFA-1 for its ligands, as detected by its increased ability to bind ICAM-1 in solution (Stewart et al., 1996). Expression of the epitope recognised by mAb 24 is dependent on temperature and Mg^{2+} or Mn^{2+} , and is a distinctive feature of this high affinity LFA-1 (Dransfield and Hogg, 1989; Stewart et al., 1996).

It is not known whether LFA-1 uses different sites to bind ligand depending on the method of activation. Sites involved in ligand binding have been identified in both the LFA-1 α subunit N-terminal repeats (Stanley et al., 1994) and in the β subunit (Bajt et al., 1995). A third putative site was initially identified when cloning of the LFA-1 α subunit revealed an insertion of approximately 200 amino acids

between repeats II and III in the N-terminus (Larson et al., 1989). It was noted that this domain is homologous to domains in von Willebrand Factor that had been shown to interact with ligand (Larson et al., 1989). This region was termed the “I” (inserted/interactive) domain. A number of pieces of evidence demonstrate that the LFA-1 I domain is indeed a major binding site for ICAM-1: anti-LFA-1 α chain antibodies that block ligand binding map to the I domain (Champe et al., 1995; Edwards et al., 1998; Randi and Hogg, 1994); the isolated I domain can bind directly to ligand (Randi and Hogg, 1994); LFA-1 in which the I domain is replaced with the murine sequence, or from which the I domain has been removed, does not bind ligand (Edwards et al., 1995; Leitinger and Hogg, 2000).

In this chapter, recombinant I domain and constituent peptides have been used to investigate how the LFA-1 I domain participates in Mg^{2+} /EGTA- and PdBu-stimulated T cell adhesion to ICAM-1 and ICAM-3.

4.2 RESULTS

4.2.1 The LFA-1 I domain inhibits Mg^{2+} /EGTA- but not PdBu-induced adhesion of T cells to ICAM-1

To investigate the role of the LFA-1 I domain in T cell adhesion to immobilised ICAM-1, a recombinant chimeric protein composed of the I domain residues Gly¹²⁸-Ser³²⁷ connected to the Fc portion of human IgG₁ (I-Fc) was tested for its effect on Mg^{2+} /EGTA- and PdBu-stimulated adhesion. I-Fc inhibited Mg^{2+} /EGTA-stimulated adhesion in a dose dependent manner, but had no effect on PdBu-stimulated adhesion (Fig. 4.1 A). Both forms of adhesion were completely blocked by mAbs against LFA-1 (Fig. 4.1 A). I-Fc with the mutation Asp²³⁹/Ala, which has been shown to be non-functional (Edwards et al., 1995; Kamata et al., 1995), did not interfere with Mg^{2+} /EGTA-stimulated adhesion (Fig. 4.1 B), suggesting that the inhibitory effect of I-Fc was specific.

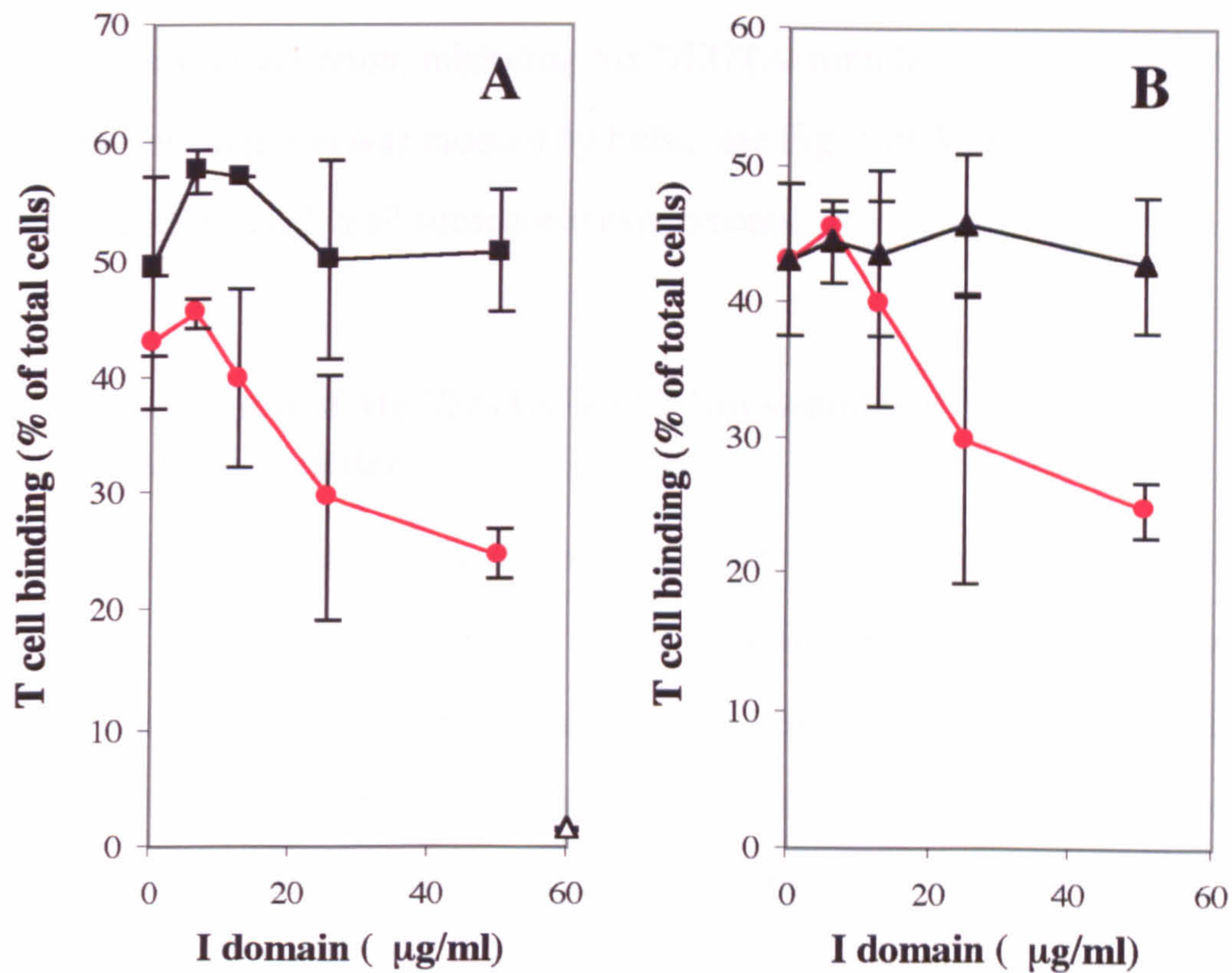


Figure 4.1 The effect of the I domain on T cell adhesion to ICAM-1

A: T cell binding to immobilised ICAM-1Fc stimulated by Mg²⁺/EGTA (●) or PdBu (■) in the presence of 0-50 μg/ml I-Fc. Anti-LFA-1 mAb 38 at 10 μg/ml (Δ) totally inhibits adhesion. **B:** Mg²⁺/EGTA stimulated T cell adhesion to ICAM-1Fc in the presence of 0-50 μg/ml wild type I-Fc (●) or D²³⁹/A I-Fc (▲). Data (mean of triplicates ± S.D.) from one representative experiment (n=3) are shown.

The I domain with an amino-terminal extension of 17 amino acids (residues Leu¹¹¹-Ser³²⁷) was made as a GST fusion protein (I-GST) (Stanley and Hogg, 1998) and proved to be more stable and more effective than I-Fc. I-GST was similar to I-Fc in its effect on adhesion, inhibiting Mg²⁺/EGTA-stimulated adhesion but having no effect when adhesion was induced by PdBu (see Fig. 1 of McDowall et al., 1998), and was therefore used in all subsequent experiments.

4.2.2 The strengths of Mg²⁺/EGTA- and PdBu-stimulated T cell adhesion to ICAM-1 are similar

The failure of the I domain to inhibit PdBu-stimulated adhesion could be explained if the overall strength of PdBu-induced adhesion was stronger, and therefore less easily inhibited, than that stimulated by Mg²⁺/EGTA. This possibility was investigated by titrating the ICAM-1 function blocking mAb 15.2 in PdBu- and Mg²⁺/EGTA-activated T cell adhesion to ICAM-1 (Fig. 4.2). Both forms of adhesion were equally inhibited by this mAb, indicating that the overall strengths of PdBu- and Mg²⁺/EGTA-stimulated adhesion are similar and that there must be an alternative explanation for the effects of the I domain being dependent on the stimulus for adhesion.

4.2.3 The LFA-1 I domain inhibits expression of the mAb 24 activation epitope on T cell LFA-1

Another mechanism by which the recombinant I domain could exert an inhibitory effect on adhesion stimulated by Mg²⁺/EGTA, but not on PdBu-stimulated adhesion, could be by preventing a conformational change necessary for the formation of the Mg²⁺/EGTA-induced high affinity form of LFA-1. To investigate this potential mechanism, the effect of the I domain on the binding of mAb 24 was tested. The epitope for this antibody is exposed upon Mg²⁺/EGTA activation of LFA-1, but not upon PdBu activation, and is thought to reflect a conformational

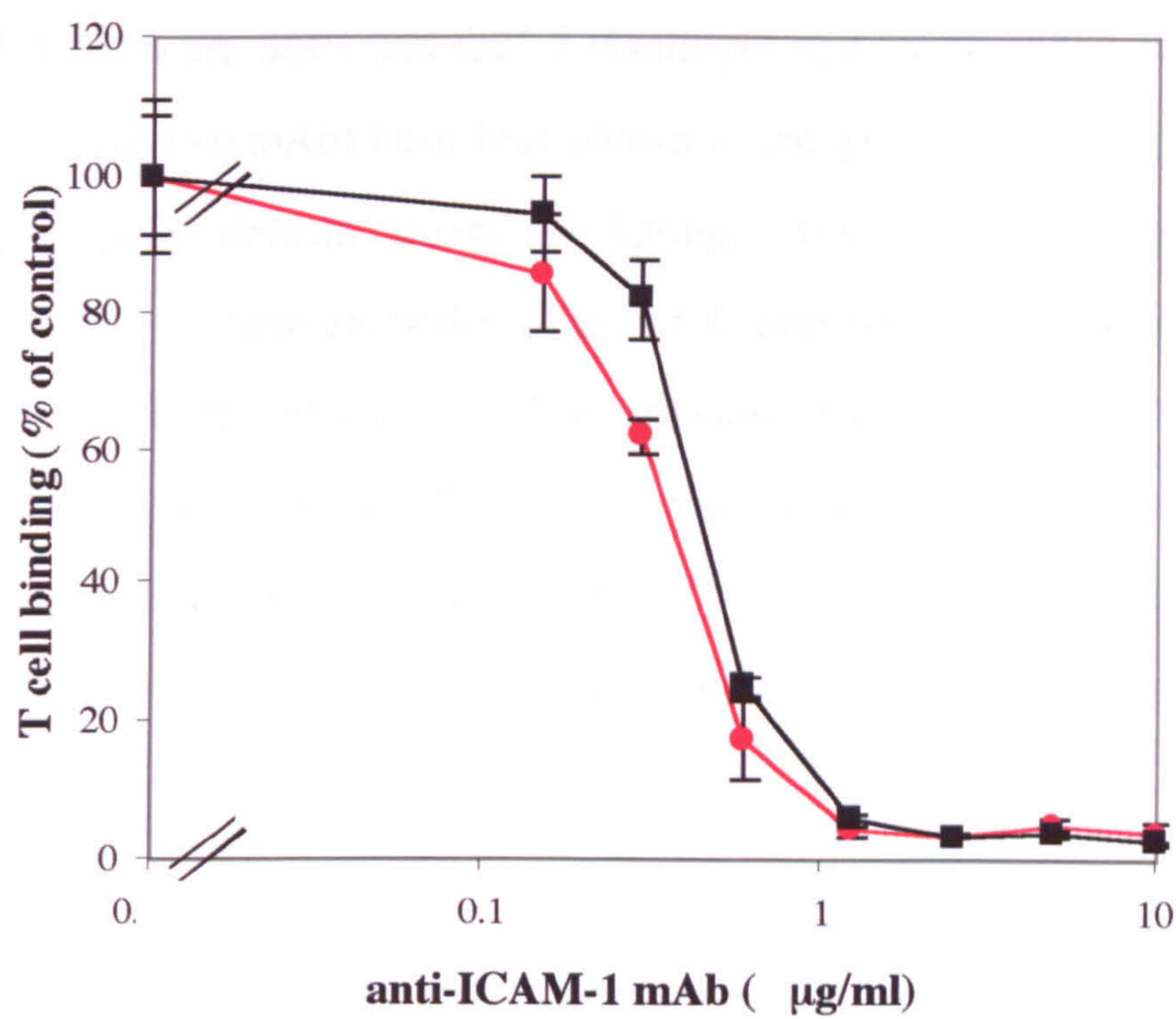


Figure 4.2 Titration of an anti-ICAM-1 mAb to compare the strength of Mg²⁺/EGTA- and PdBu-stimulated adhesion
T cell binding to immobilised ICAM-1Fc stimulated by Mg²⁺/EGTA (●) or PdBu (■) in the presence of 0-10 μg/ml anti-ICAM-1 mAb 15.2. Data (mean of triplicates ± S.D.) from one representative experiment (n=4) are shown.

change which leads to the high affinity form of the integrin (Stewart et al., 1996). I-GST inhibited the expression of the mAb 24 epitope in a dose dependent manner (Fig. 4.3 A and B). Two other anti-LFA-1 α subunit mAbs which, like 24, do not bind to the I domain are S6F1 and G25.2 (Leitinger and Hogg, 2000; Randi and Hogg, 1994). These two mAbs have been shown to recognise different epitopes in the putative β -propeller domain (Huang and Springer, 1995). I-GST did not affect the binding of either of these antibodies (Fig. 4.3 C and D). These data show that the I domain specifically interferes with expression of the activation dependent epitope recognised by mAb 24. This result implies that the I domain inhibits Mg^{2+} /EGTA-stimulated T cell binding to ICAM-1 by interacting with LFA-1 on the T cells, thereby preventing conversion to the high affinity conformation of the integrin.

4.2.4 LFA-1 interaction with ICAM-1 is not necessary for expression of the activation epitope 24

If mAb 24 recognises a ligand induced binding site (LIBS) on LFA-1, then the I domain could inhibit expression of the mAb 24 epitope by binding to ICAM-1, thereby blocking the interaction of ICAM-1 with LFA-1 necessary for triggering formation of the LIBS. Alternatively, although it has been shown that both I-Fc and I-GST bind to ICAM-1Fc in cell free assays (Randi and Hogg, 1994; Stanley and Hogg, 1998), in the T cell adhesion assay the I domain may bind to LFA-1 on the T cells, thus preventing a conformational change necessary for the epitope for mAb 24 to be revealed. Blocking LFA-1 interaction with ICAM-1, either with ICAM-1 mAbs 15.2 or RR1/1 or with LFA-1 mAb 38, does not alter 24 expression (Fig. 4.4), suggesting that LFA-1 interaction with ICAM-1 is not required for expression of the mAb 24 epitope. These findings imply that the I domain inhibits T cell binding by interacting with LFA-1 on the T cell and preventing a conformational change necessary for formation of high affinity LFA-1.

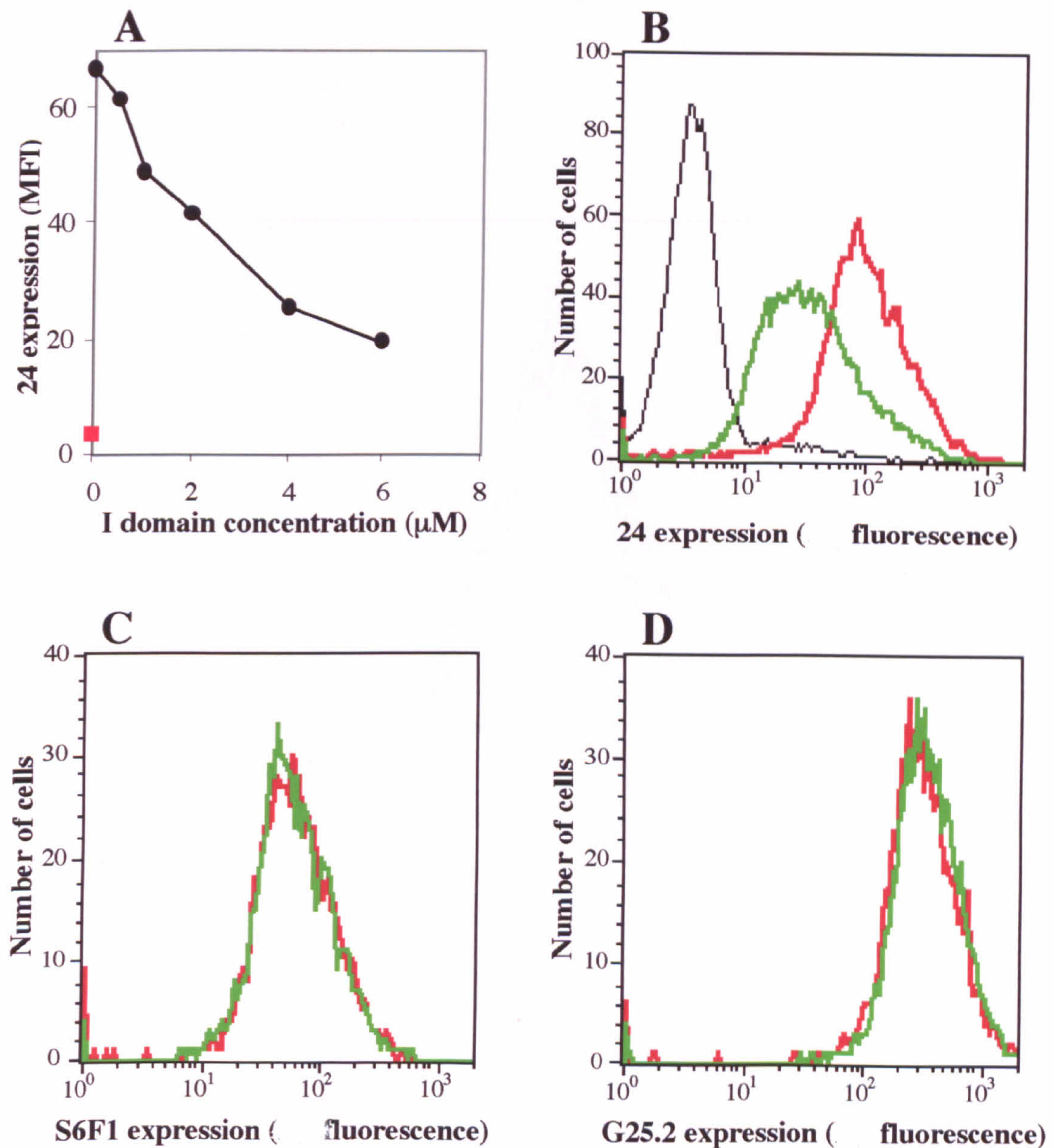


Figure 4.3 The effect of the I domain on binding of mAbs 24, S6F1 and G25.2 to T cells stimulated by $\text{Mg}^{2+}/\text{EGTA}$

A: Mean fluorescence of mAb 24 on T cells incubated at 37°C in $\text{Mg}^{2+}/\text{EGTA}$ in the presence of 0-6 μM I-GST (\bullet). The background binding of mAb 24 to T cells incubated in $\text{Mg}^{2+}/\text{EGTA}$ at 4°C (\blacksquare). Data from one representative experiment ($n=3$) are shown. **B:** The binding of mAb 24 to T cells incubated in $\text{Mg}^{2+}/\text{EGTA}$ at 4°C (—), at 37°C (—) and at 37°C with 4 μM I-GST (—). Data from one representative experiment ($n=4$) are shown. The binding of **C:** mAb S6F1 and **D:** mAb G25.2 to T cells incubated in $\text{Mg}^{2+}/\text{EGTA}$ at 37°C (—) and at 37°C with 4 μM I-GST (—). Data from one representative experiment ($n=2$) are shown.

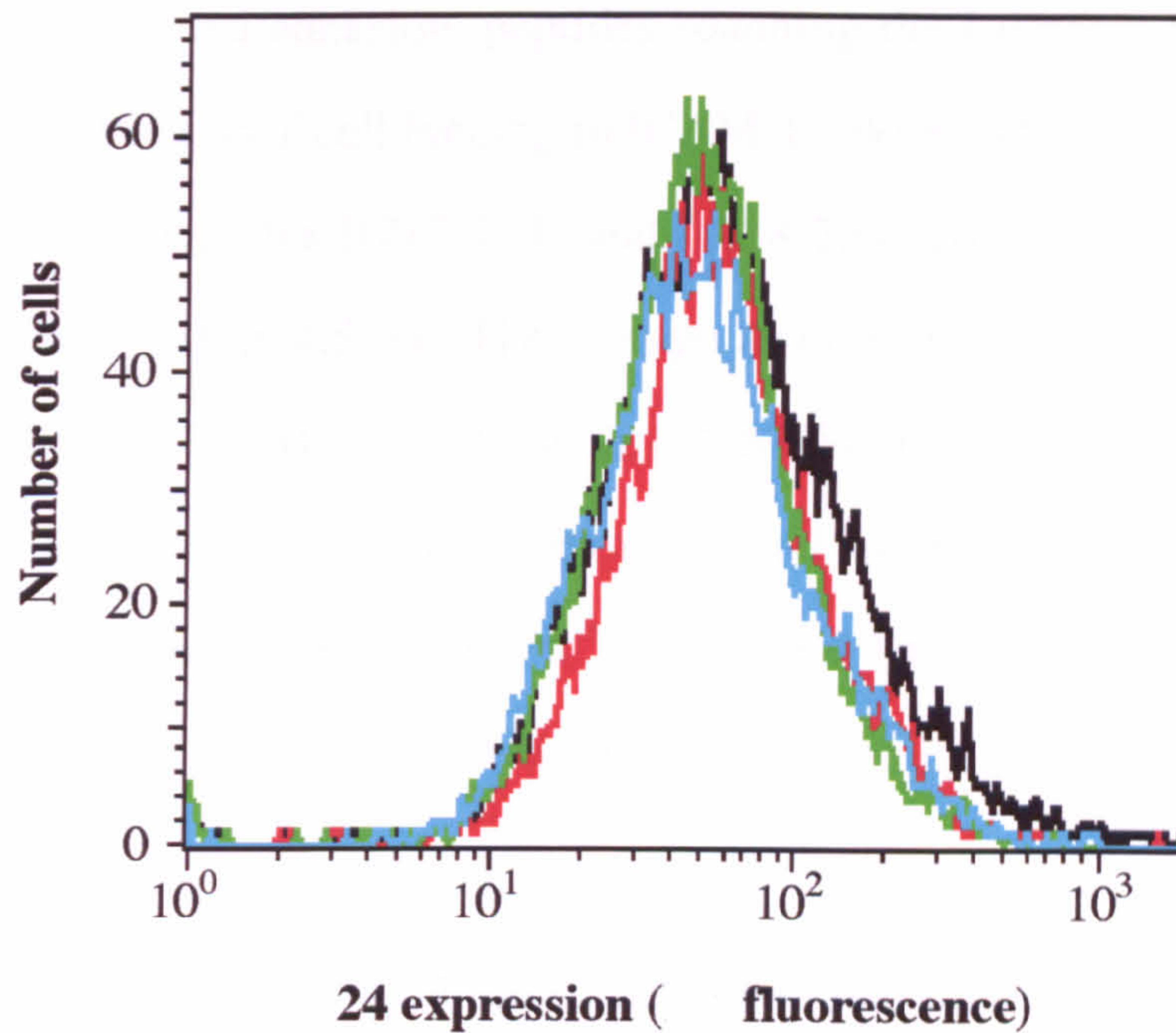


Figure 4.4 The effect of inhibiting LFA-1 interaction with ICAM-1 on binding of mAb 24

The binding of mAb 24 to T cells incubated at 37°C in Mg^{2+} /EGTA alone (—), or in the presence of 10 μ g/ml anti-LFA-1 mAb 38 (—), or anti-ICAM-1 mAbs 15.2 (—) or RR1/1 (—). Data from one representative experiment (n=3) are shown.

4.2.5 Peptides I(217-233) and I(238-254) inhibit Mg^{2+} /EGTA- but not PdBu-stimulated T cell binding to ICAM-1

To determine the regions within the I domain specifically required for Mg^{2+} /EGTA-stimulated adhesion, peptides spanning the I domain (Table 4.1) were tested for their effect on T cell binding to ICAM-1. When adhesion was activated by Mg^{2+} /EGTA only peptides I(217-233) and I(238-254) consistently blocked LFA-1 binding to ICAM-1 (Fig. 4.5 A). The average level of inhibition with 2 mM peptide was 50% with I(217-233) and 72% with I(238-254) (n=13). When adhesion was induced with PdBu none of the peptides tested had an inhibitory effect (Fig. 4.5 B). These results indicate that residues Thr²¹⁷-Val²³³ and Thr²³⁸-Ile²⁵⁴ of the I domain are specifically involved in Mg^{2+} /EGTA-induced LFA-1 binding to ICAM-1.

4.2.6 Subpeptides of I(217-233) and I(238-254) also inhibit Mg^{2+} /EGTA-stimulated T cell binding to ICAM-1

To further define the residues of the I domain critical for Mg^{2+} /EGTA-stimulated LFA-1 binding to ICAM-1 overlapping peptides covering the region Thr²¹⁷-Ile²⁵⁴ were made (Table 4.1 - second series). I(238-246), I(242-254) and I(247-258), sub-peptides of I(238-254), the most inhibitory peptide in the first series, all blocked Mg^{2+} /EGTA-stimulated T cell binding to ICAM-1 in a dose dependent manner equivalent to the parent peptide (Fig. 4.6 A). The control peptide I(256-275) had no effect at the same concentrations (Fig. 4.6 A). I(223-233) also had an inhibitory effect on adhesion (Fig. 4.6 B) which corresponded to the levels of inhibition with the parent peptide I(217-233) (data not shown). Unfortunately I(210-223) could not be tested due to its limited solubility.

Scrambled versions of I(223-233) and I(238-254) were made (Table 4.1) and these controls did not affect T cell binding to ICAM-1, indicating that the dose-dependent inhibition seen with I(223-233) and I(238-254) was specific (Fig. 4.6 B).

Peptide	Amino acid sequence
I(130-159)	VDLVFLFDGS.MSLQPDEFQK.ILDFMKDVMK
I(193-202)	DALLKHVKHM
I(217-233)	TEVFREEELGA.RPDATKV
I(238-254)	TDGEATDSGN.IDAAKDI
I(256-275)	RYIIGIGKHF.QTKESQETLH
I(276-295)	KFASKPASEF.VKILDTFEKL
I(296-312)	KDLFTELQKK.IYVIEGT
Second series	
I(223-233)	ELGARPDATK.V
I(238-246)	TDGEATDSG
I(242-254)	ATDSGNIDAA.KDI
I(247-258)	NIDAAKDIIR.YI
Scrambled controls	
I(223-233)	VKLETAGADR.P
I(238-254)	DIDTKAEGAD.TAINSDG

Table 4.1 LFA-1 I Domain Peptides and Amino Acid Sequences

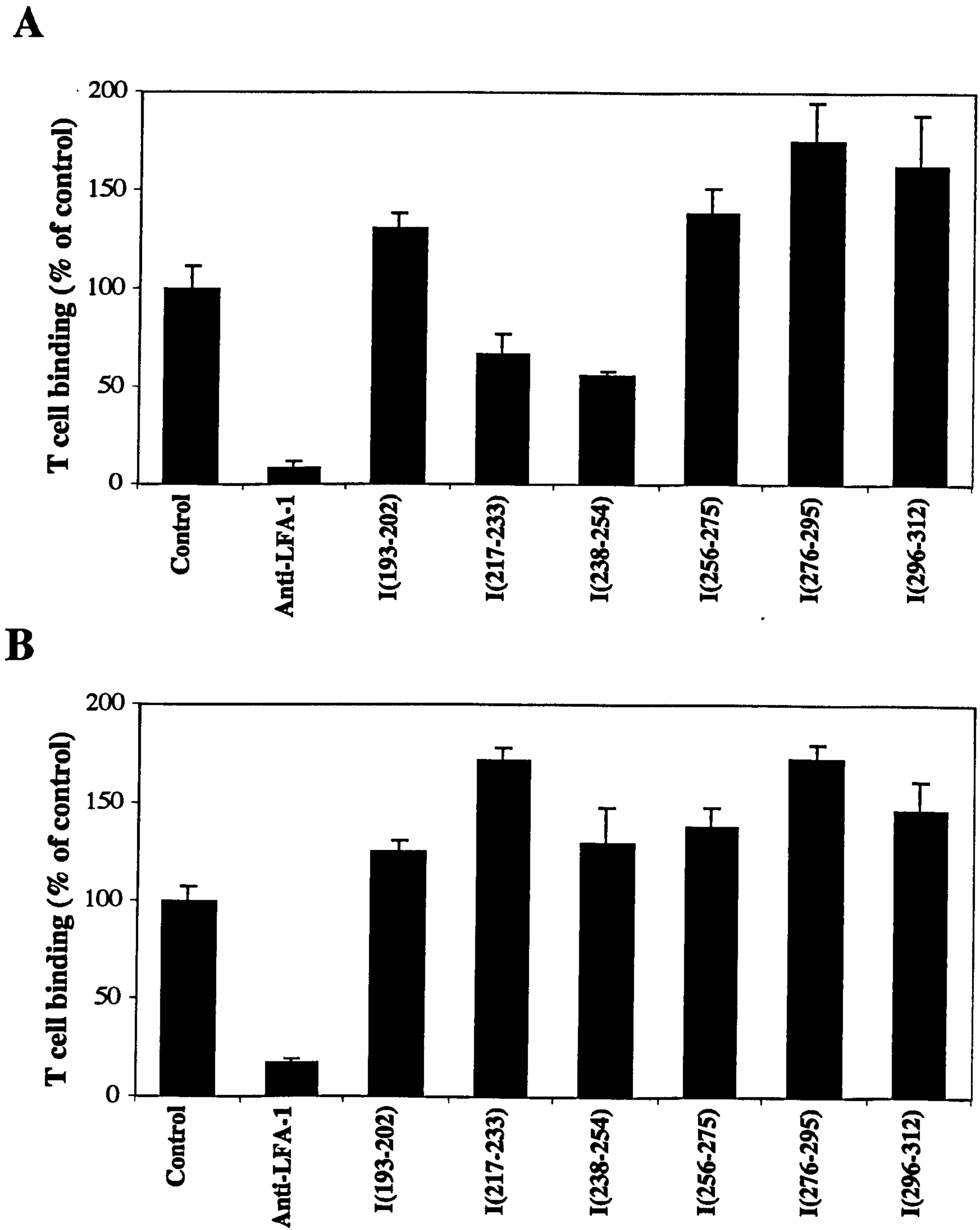


Figure 4.5 The effect of LFA-1 I domain peptides on T cell adhesion to ICAM-1.

The effect of LFA-1 peptides (2 mM) on T cell binding to ICAM-1Fc following stimulation with **A:** Mg^{2+} /EGTA or **B:** PdBu. Data (mean of triplicates \pm S.D.) from one experiment (n=13) are shown.

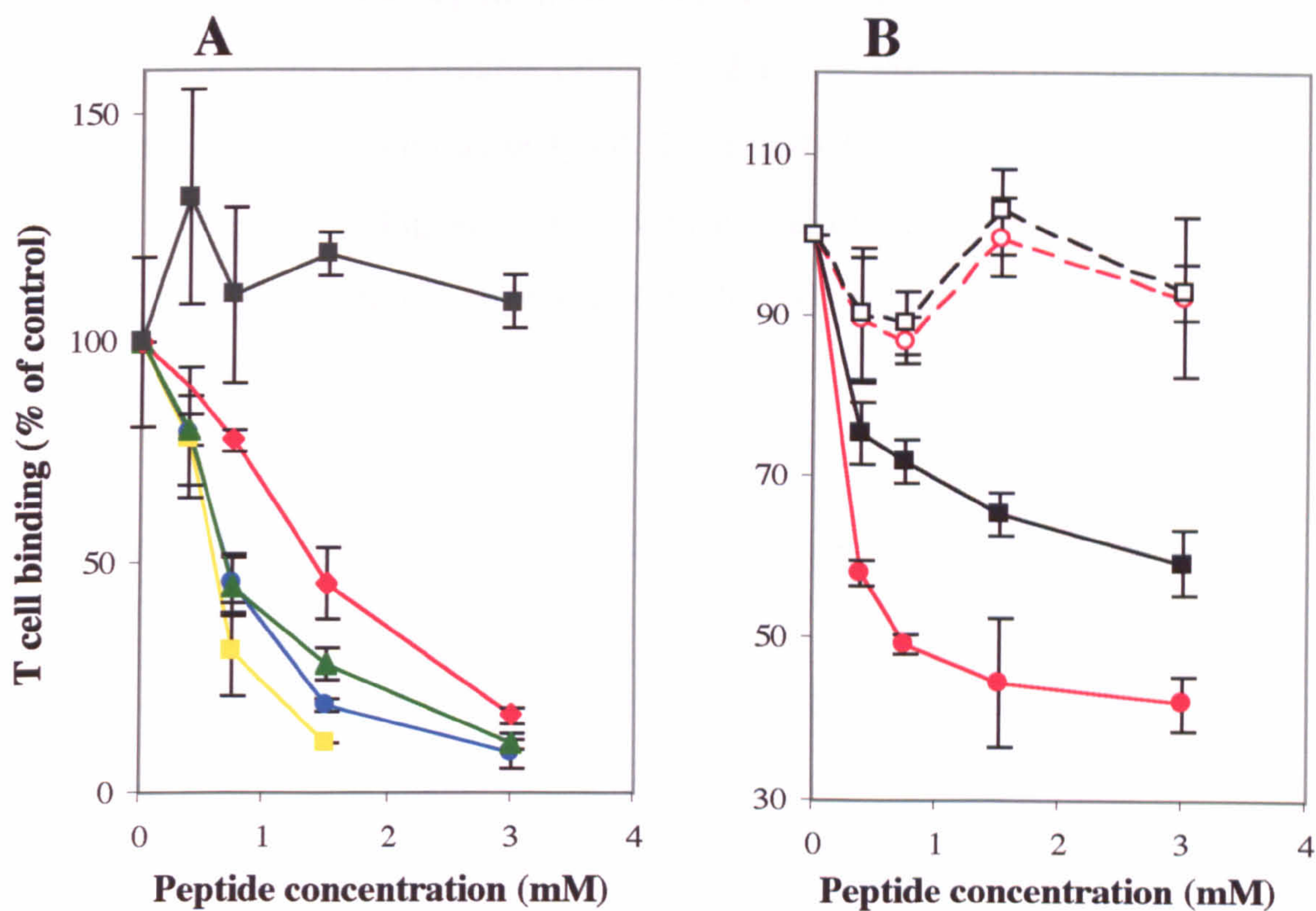


Figure 4.6 $Mg^{2+}/EGTA$ stimulated T cell adhesion to ICAM-1 with a titration of inhibitory LFA-1 I domain peptides and control peptides

$Mg^{2+}/EGTA$ stimulated T cell binding to ICAM-1Fc with **A:** titration of I(238-254) (♦), overlapping peptides I(238-246) (■), I(242-254) (●), I(247-258) (▲) and control peptide I(256-275) (■) and **B:** titration of I(223-233) (■), its scrambled control (□), I(238-254) (●) and its scrambled control (○). Data (mean of triplicates \pm S.D.) from one representative experiment (n=3) are shown.

4.2.7 Peptide I(238-254) inhibits 24 expression

To test whether the peptide inhibition of T cell binding to ICAM-1 occurs by the same mechanism as the soluble I domain inhibition, the effect of peptide I(238-254) on mAb 24 expression was analysed. I(238-254), but not its scrambled control, inhibited 24 expression (Fig. 4.7). The average level of inhibition of 24 expression was 55% (n=4). These results suggest that I(238-254) and the I domain interact with T cells in a similar manner.

4.2.8 The position of I(223-233) and I(242-254) on the LFA-1 I domain crystal structure

When the two function blocking peptides, I(223-233) and I(242-254), are superimposed on the crystal structure of the LFA-1 I domain (Qu and Leahy, 1995) they span two adjacent areas. The sequence Glu²²³-Val²³³ corresponds to the loop after the α_4 helix which terminates in the β_3 strand and Ala²⁴²-Ile²⁵⁴ corresponds to the β_3 to α_5 loop and continues as far as the end of the α_5 helix (Fig. 4.8).

4.2.9 Peptide I(256-275) inhibits Mg²⁺/EGTA-stimulated binding to ICAM-3 but not to ICAM-1

The first series of peptides (Table 4.1) were also tested for their effect on Mg²⁺/EGTA-stimulated T cell adhesion to ICAM-3. As for ICAM-1, peptides I(217-233) and I(238-254) inhibited adhesion but, interestingly, I(256-275) also had an inhibitory effect (Fig. 4.9). The average level of adhesion in the presence of I(256-275) was 30% to ICAM-3 (n=4) and 119% to ICAM-1 (n=4). This result supports the idea that LFA-1 binds to ICAM-1 and ICAM-3 in a similar, but not identical, manner.

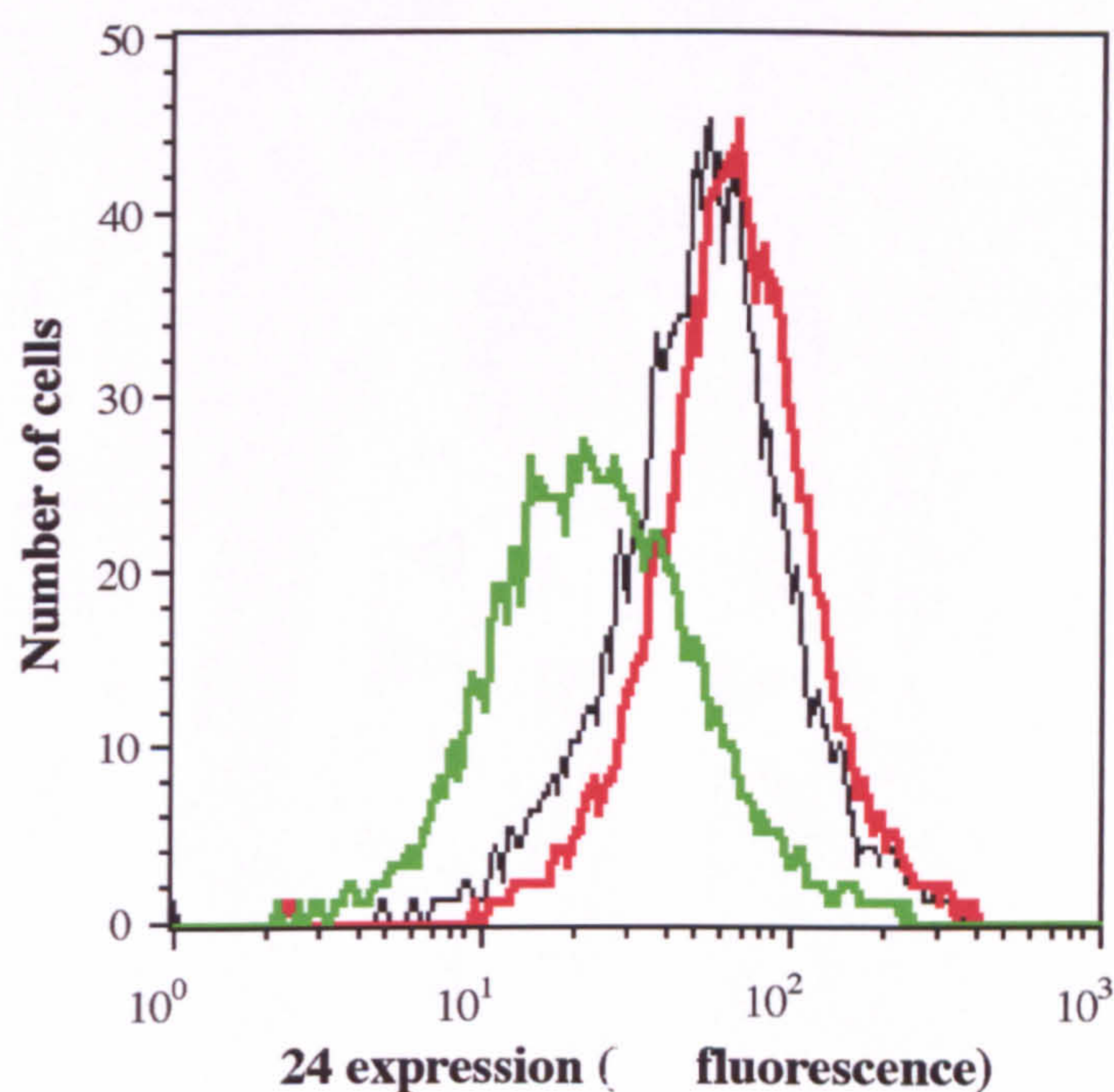


Figure 4.7 The effect of peptide I(238-254) on the expression of the mAb 24 epitope

The expression of the mAb 24 epitope on T cells incubated at 37°C in Mg^{2+} /EGTA either without peptide (—), or with 2 mM I(238-254) (—) or its scrambled control (—). Data from one representative experiment (n=4) are shown.

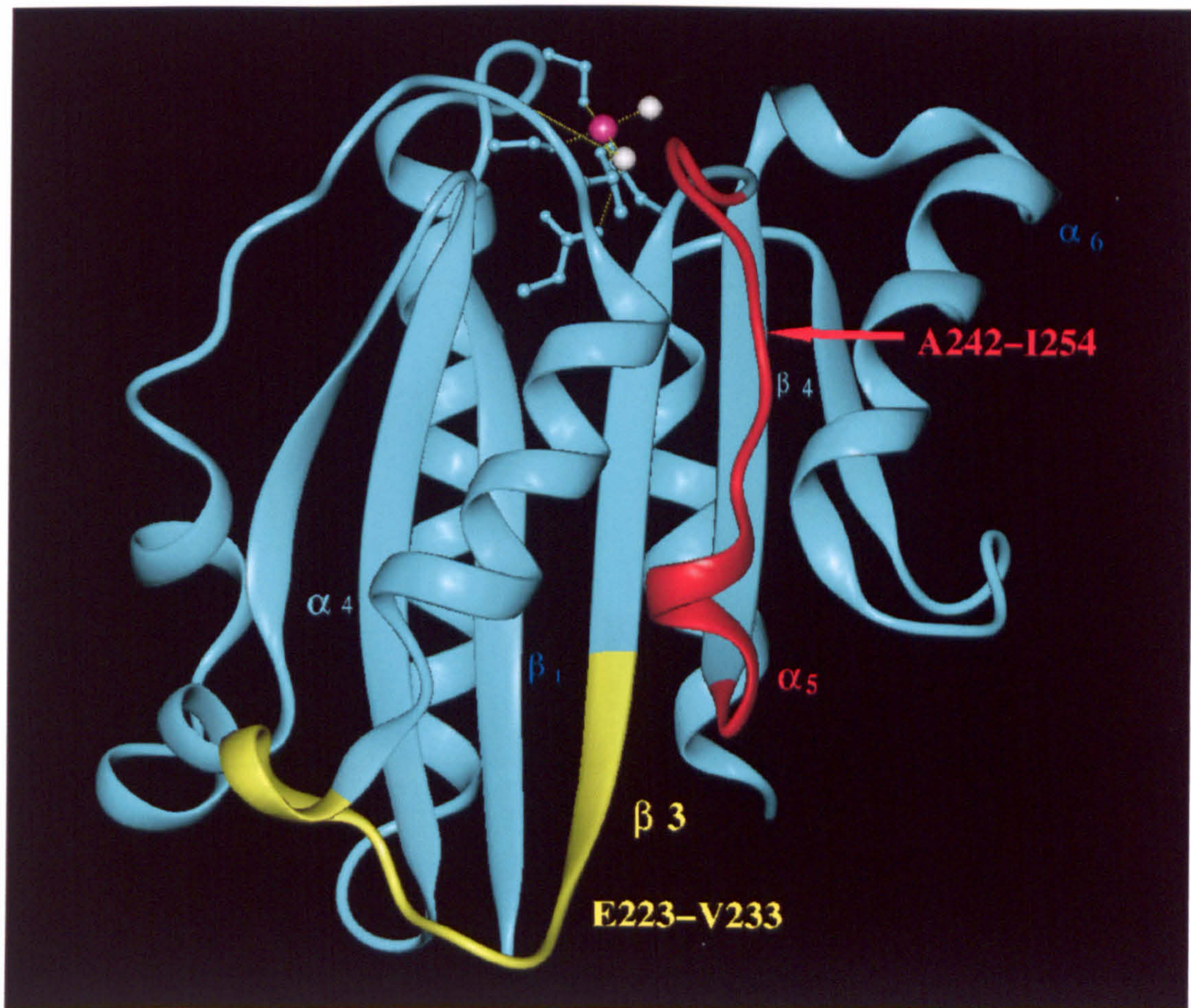


Figure 4.8 The LFA-1 I domain crystal structure indicating the position of the peptides that inhibit LFA-1 interaction with ICAM-1

A ribbon diagram of the LFA-1 I domain with the sequence corresponding to the I(223-233) peptide indicated in yellow and I(242-254) in red. The metal ion bound to the MIDAS motif is shown in pink and the water molecules in white. This figure was generated by P. Bates, Biomolecular Modelling Laboratory, ICRF using coordinates obtained from the Brookhaven Data Bank.

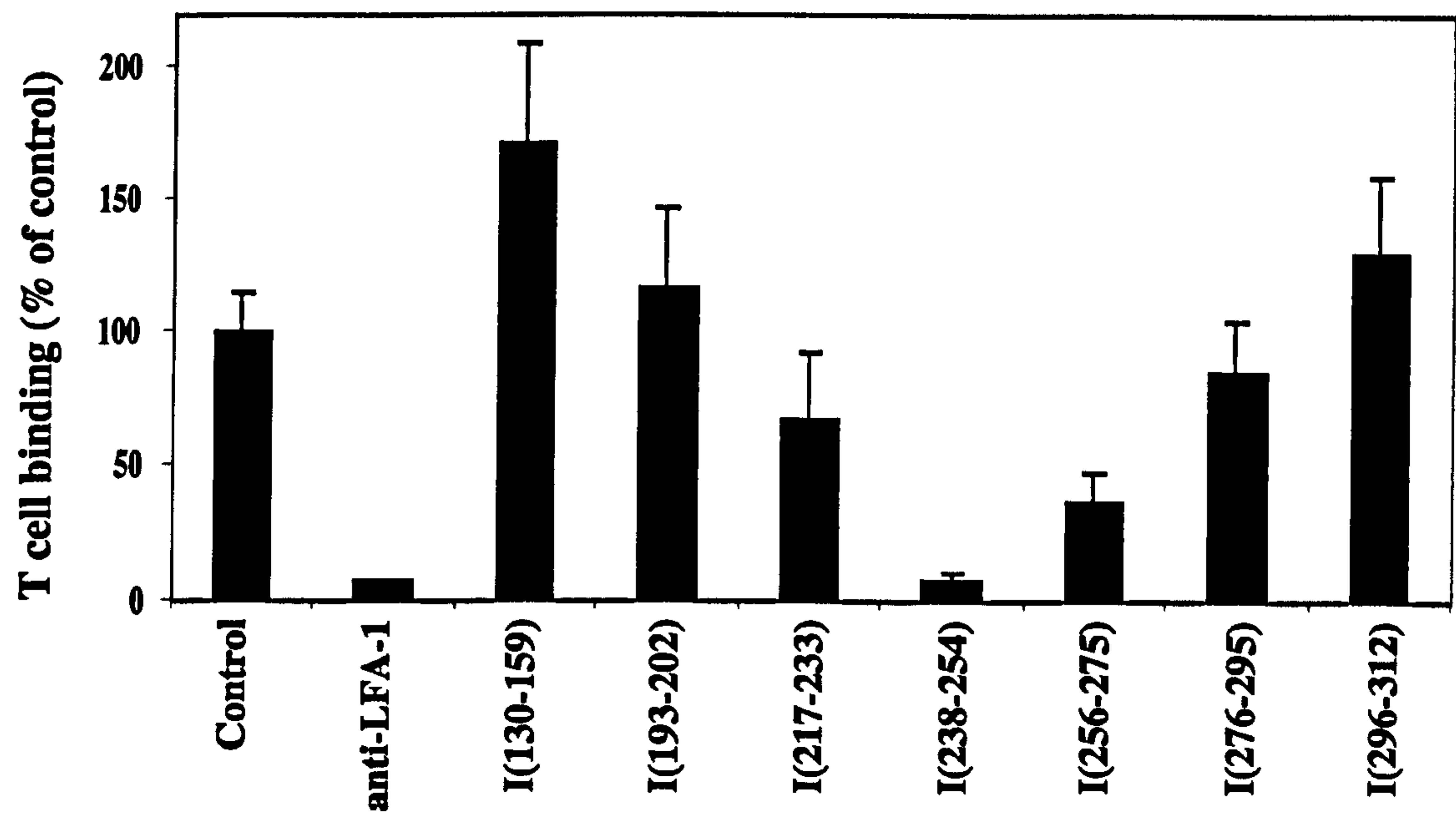


Figure 4.9 The effect of LFA-1 I domain peptides on Mg^{2+} /EGTA stimulated T cell adhesion to ICAM-3

T cell binding to ICAM-3Fc stimulated by Mg^{2+} /EGTA in the presence of LFA-1 I domain peptides (2 mM). Data (mean of triplicates \pm S.D.) from one experiment (n=4) are shown.

4.2.10 PdBu-stimulated T cell adhesion to ICAM-1 is more sensitive to inhibition by anti-CD18 mAbs than Mg^{2+} /EGTA-stimulated adhesion

A panel of anti-CD18 (LFA-1 β_2 subunit) mAbs were tested for their effect on T cell adhesion to ICAM-1. It was found that some CD18 mAbs are more potent inhibitors of PdBu-stimulated binding than of Mg^{2+} /EGTA-stimulated binding (Fig. 4.10 A). A number of the mAbs were titrated. Although the LFA-1 α subunit mAb 38 inhibited Mg^{2+} /EGTA- and PdBu-stimulated binding to a similar extent at all concentrations (Fig. 4.10 B), it was found that the β_2 subunit mAbs TS1/18 and GRF1 inhibited PdBu-stimulated binding to a greater extent than Mg^{2+} /EGTA-stimulated binding (Fig. 4.10 D and E). This effect was not seen with all β_2 subunit mAbs, however, as the mAb 6.5E had similar effects on both forms of adhesion (Fig. 4.10 C). These results suggest that the LFA-1 β subunit has a more prominent role in PdBu-stimulated adhesion than in the high affinity Mg^{2+} /EGTA-induced LFA-1 adhesion.

4.3 DISCUSSION

The finding that recombinant LFA-1 I domain interfered with Mg^{2+} /EGTA-activated, but not PdBu-activated, T cell adhesion to ICAM-1 implies that the I domain has a distinct role in high affinity LFA-1 adhesion. An alternative explanation for this result was that Mg^{2+} /EGTA-stimulated adhesion was stronger, and therefore less easily inhibited, than PdBu-stimulated adhesion. Both forms of adhesion were equally inhibited by an anti-ICAM-1 mAb, however. To further discount this possibility, quantitative centrifugal removal assays were performed in which the number of cells remaining bound to ICAM-1 following subjection to increasing relative centrifugal forces was measured. The number of cells was equal for both forms of adhesion (McDowall et al., 1998), therefore, together these data confirm that the overall strengths of Mg^{2+} /EGTA- and PdBu-stimulated adhesion are

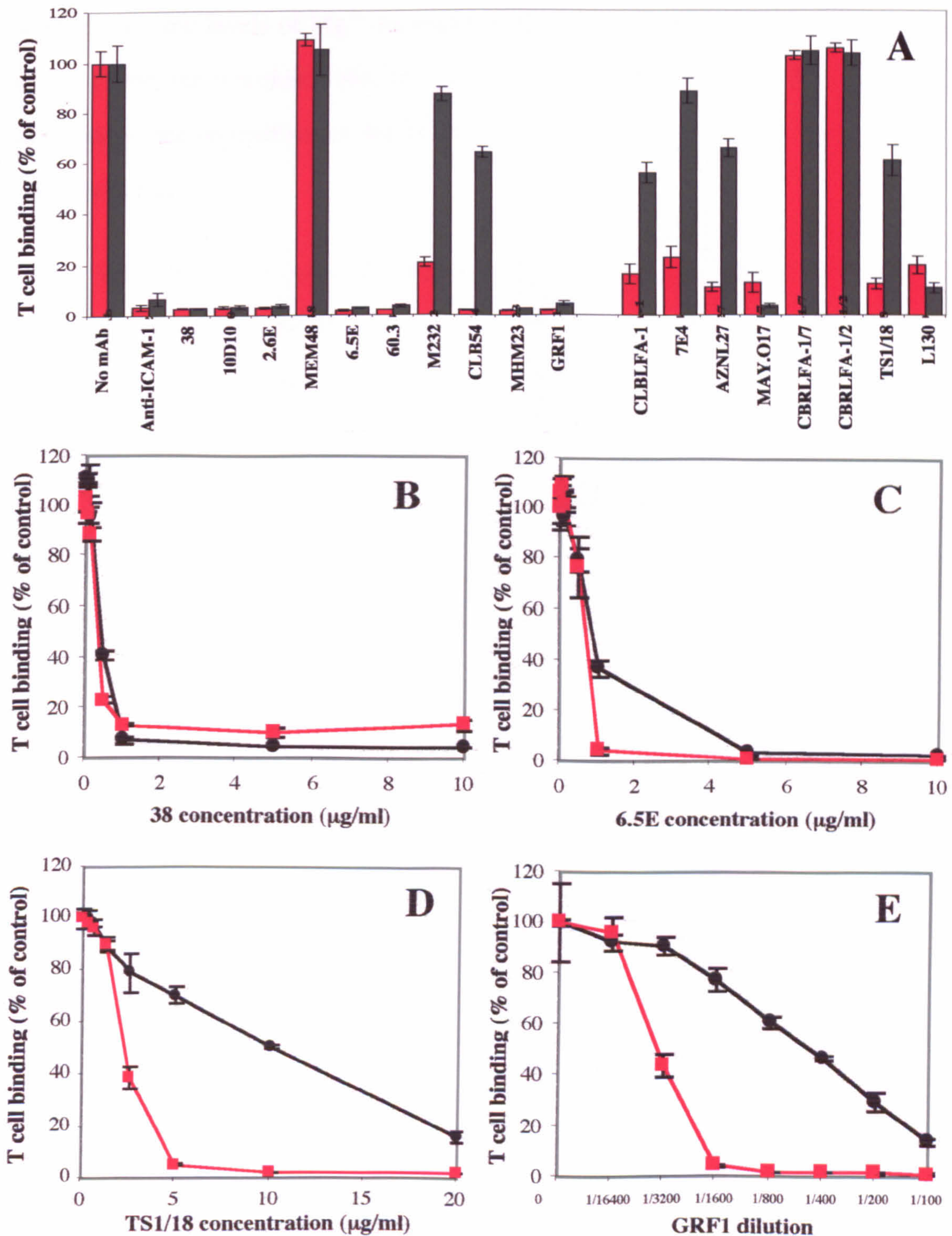


Figure 4.10 The effect of CD18 mAbs on T cell binding to ICAM-1.

A: T cell binding to ICAM-1Fc stimulated by 50 nM PdBu (■) or 2 mM Mg²⁺/1 mM EGTA (■) in the presence of the indicated mAbs at 5 μg/ml or 1/100 ascites for GRF1 and CLB54 (n=3). T cell binding to ICAM-1Fc stimulated by 50 nM PdBu (■) or 2 mM Mg²⁺/1 mM EGTA (●) in the presence of **B:** 0-10 μg/ml CD11a mAb 38 (n=4), **C:** 0-10 μg/ml CD18 mAb 6.5E (n=3), **D:** 0-20 μg/ml TS1/18 (n=5), **E:** 1/100-1/16400 dilution of CD18 mAb GRF1 (n=3). Data (mean of triplicates ± S.D.) from one representative experiment are shown.

similar. As low levels of Mg^{2+} are essential for PdBu-stimulated adhesion (Stewart et al., 1996), the possibility that the selective inhibition of Mg^{2+} /EGTA-stimulated adhesion by the recombinant I domain was due to chelation of Mg^{2+} by the I domain was discounted.

With the use of mAb 24 it was determined that the inhibitory effect of the I domain occurs via interaction with T cell LFA-1 rather than with the immobilised ICAM-1. The expression of the epitope recognised by mAb 24 is one feature which distinguishes the Mg^{2+} /EGTA-induced form of LFA-1 from the resting and PdBu-induced forms (Stewart et al., 1996). Exposure of this epitope is thought to reflect a conformational change induced by Mg^{2+} binding to LFA-1. The inhibition of 24 expression by the recombinant I domain therefore suggests that the recombinant I domain is interfering with a conformational change within LFA-1 required for formation of the high affinity form of the integrin. It is known that the 24 epitope is somewhere on the LFA-1 α subunit other than the I domain (Dransfield and Hogg, 1989; Leitinger and Hogg, 2000; Randi and Hogg, 1994). This suggests that what is being measured by 24 expression is not a conformational change in the I domain itself, although recent evidence shows this does occur (Emsley et al., 2000; Oxvig et al., 1999; Shimaoka et al., 2000), but an interdomain movement involving the I domain.

Peptides covering a region of the I domain including the α_4 to β_3 loop, the β_3 - α_5 loop and the α_5 helix mimicked the behaviour of recombinant I domain in preventing Mg^{2+} /EGTA-stimulated adhesion, and for I(238-254) in preventing mAb 24 expression. The I domain crystal structure shows that these two regions lie on the same face of the domain (see Fig. 4.8). Evidence suggests that ligand binds to the MIDAS face of I domains (Edwards et al., 1998; Huang and Springer, 1995; Käpylä et al., 2000; Rieu et al., 1996; Smith et al., 2000a; Zhang and Plow, 1997). The sequences corresponding to the peptides I(223-233) and I(242-254) lie on the side and towards the opposite end of the domain to the MIDAS face. It therefore seems

unlikely that the inhibitory effect of these two peptides is due to direct blocking of ligand binding. Lovastatin, a fungal metabolite, is an inhibitor of LFA-1/ICAM-1 interaction (Kallen et al., 1999). It binds to a pocket formed by the α_1 and α_7 helices and the β_4 and β_5 strands on the opposite face of the I domain to the MIDAS, and is therefore thought to inhibit by an indirect mechanism. It is proposed that this pocket is a regulatory site for LFA-1 activation and that lovastatin binding locks LFA-1 in an inactive form, thereby allosterically interfering with ligand binding (Kallen et al., 1999). The peptides I(223-233) and I(242-254) lie close to this pocket, therefore may inhibit interaction with ICAM-1 by a mechanism similar to that proposed for lovastatin.

The seven N-terminal repeat sequences of a typical integrin α subunit have been modelled as a β propeller fold homologous to the heterotrimeric G protein β subunit (Sondek et al., 1996; Springer, 1997). The I domain, which is structurally homologous to the G protein α subunit, can be positioned on the upper surface of the β -propeller, to one side of the central axis, mimicking the relationship between the G protein α and β subunits (Lambright et al., 1996; Springer, 1997). Looking down on the central axis of the β -propeller (see Fig. 4.11), the Ala²⁴²-Ile²⁵⁴ sequence protrudes over the central cavity, and, potentially, the Glu²²³-Val²³³ sequence makes contact with the β -propeller domain. One speculation is that the conformational change in LFA-1 that occurs upon Mg²⁺ activation involves movement between the I domain and the β -propeller. Therefore, recombinant I domain and peptides representing the α_4 and α_5 loop regions could compete with cellular I domain in intact LFA-1 for binding to the β -propeller domain, thereby physically blocking activation.

It was of interest that peptide I(256-275) interfered with LFA-1 binding to ICAM-3 without having an effect on binding to ICAM-1. This peptide covers the β_4 strand, the β_4 to α_6 loop and most of the α_6 helix. These regions are indicated in Figure 4.8 and lie close in the structure to the sequences represented by I(223-233) and I(242-254). Whether this peptide inhibits 24 expression remains to be tested,



Figure 4.11 A model of the association between the LFA-1 I domain and a β -propeller domain

A ribbon diagram of the LFA-1 I domain (blue) and the β -propeller domain of the heterotrimeric G protein transducin (green) in the same relative orientation as the α and β subunits of transducin (Lambright et al., 1996). The sequence corresponding to the I domain peptide I(223-233) is indicated in yellow and I(242-254) in red. The arrow points to the MIDAS motif. This model was generated by P. Bates, Biomolecular Modelling Laboratory, ICRF using coordinates taken from the Brookhaven Data Bank.

therefore at present the mechanism by which this peptide inhibits binding to ICAM-3 cannot be ascertained. There is evidence from the use of anti-LFA-1 I domain mAbs that ICAM-1 and ICAM-3 bind the I domain at distinct sites (Landis et al., 1994). Point mutation of LFA-1 residues Lys¹²⁷ and Asn¹²⁹ abrogates adhesion to ICAM-3, without affecting binding to ICAM-1 (van Kooyk et al., 1996). Although shorter peptides covering these residues have no effect, a peptide spanning residues Gly¹¹⁸-Gly¹³⁸ inhibits LFA-1 binding to ICAM-3 but not to ICAM-1 (van Kooyk et al., 1996), indicating that the sequences surrounding Lys¹²⁷ and Asn¹²⁹ are important for the effect of the peptide. Residues Gly¹¹⁸-Gly¹³⁸ start N-terminal to the I domain and extend to the end of the β_1 strand, therefore are not in close proximity to residues covered by peptide I(256-275). Unfortunately the Gly¹¹⁸-Gly¹³⁸ peptide proved to be insoluble in our hands, therefore could not be tested.

The fact that recombinant I domain does not block LFA-1 activation by PdBu implies that PdBu stimulation does not involve a conformational change involving the I domain. This is in keeping with the fact that PdBu does not cause an affinity alteration or a detectable conformational change in LFA-1 (Stewart et al., 1996). This does not preclude the involvement of the I domain of intact LFA-1 in ligand binding following PdBu stimulation. It was intriguing to find that PdBu-stimulated adhesion was more sensitive than Mg²⁺/EGTA-stimulated adhesion to inhibition by mAbs against the LFA-1 β subunit. The expression of these mAbs is not influenced by the state of LFA-1 activation (data not shown), therefore other explanations for these results should be explored. These mAbs might prevent a conformational change required for PdBu-stimulated adhesion, although, as mentioned above, such a change has not been detected. A second possibility is that the mAbs could block a ligand binding site used by the PdBu-stimulated form only, however, as discussed earlier, there is no evidence for the different forms of LFA-1 having different binding sites on ICAM-1. Finally, the mAbs might inhibit the clustering that is required for PdBu-stimulated adhesion (see Chapter 5). The epitopes for most of the LFA-1 β

subunit mAbs tested in this study have been mapped (Huang et al., 1997). All the mAbs that were most effective at inhibiting PdBu-stimulated binding map to the first and last α -helices in the β_2 subunit I-like domain. These helices are predicted to be neighbouring. 6.5E, L130 and MAY.017 bind elsewhere in the β_2 subunit I-like domain and all appeared to inhibit both forms of adhesion equally. L130 and MAY.017 have yet to be titrated, however. KIM127, KIM185, CBRLFA-1/2 and MEM-48 map to the cysteine rich region at the C-terminal of the β_2 subunit extracellular domain and activate LFA-1 binding to ICAM-1 (data not shown), in agreement with other studies (Andrew et al., 1993; Huang et al., 1997; Huang et al., 2000; Petruzzelli et al., 1995; Stephens et al., 1995). CBRLFA-1/7, which had no effect on LFA-1 adhesion, also maps to this region (Huang et al., 2000).

From this study it can be concluded that the addition of recombinant I domain or peptides interferes with interdomain movements or alterations in intersubunit associations and thereby prevents changes in the quaternary structure of LFA-1. The results give rise to the idea that activation of LFA-1 to a high affinity form involves interdomain movement that alters the association of the I domain with the β -propeller domain or β subunit and in this way leads to structural changes that increase the affinity for ligand. Preliminary evidence is also provided to suggest that LFA-1 interaction with ICAM-1 and ICAM-3 differs, and that the LFA-1 β subunit plays different roles in adhesion depending on the method of activation.

CHAPTER 5

LFA-1 MEDIATED T CELL ADHESION IS REGULATED BY THE PROTEASE CALPAIN

5.1 INTRODUCTION

The divalent cation Ca^{2+} is a second messenger involved in many cellular processes. In resting cells, the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is maintained at 10-100 nM by Ca^{2+} pumps, which transport Ca^{2+} into the endoplasmic reticulum (ER) or out of the cell. Cell activation can increase the $[\text{Ca}^{2+}]_i$ to 500-1000 nM. Stimulation through G protein coupled receptors and receptor tyrosine kinases activates phospholipase C, which hydrolyses phosphatidylinositol 4,5,bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 diffuses rapidly from the plasma membrane to interact with an ion channel on the ER, thus causing channel activation and release of stored Ca^{2+} into the cytosol to generate the initial Ca^{2+} signal. The depletion of Ca^{2+} from the ER triggers an influx of Ca^{2+} across the plasma membrane. This second phase of the Ca^{2+} signal, termed capacitative calcium entry, replenishes the intracellular stores and produces a more sustained elevation of $[\text{Ca}^{2+}]_i$ (reviewed in (Clapham, 1995)). The mechanism by which the plasma membrane Ca^{2+} channels are activated in response to a fall in the Ca^{2+} concentration in the ER has yet to be established, although two alternative mechanisms have been proposed: release of a chemical messenger from the ER or physical interaction between the ER and the plasma membrane (reviewed in (Putney, 1999)).

In T cells, increases in $[\text{Ca}^{2+}]_i$ occur upon T cell receptor (TCR) signalling (reviewed in (van Leeuwen and Samelson, 1999)) and chemokine receptor signalling (reviewed in (Ward et al., 1998)). The raised $[\text{Ca}^{2+}]_i$ has many downstream effects

that ultimately lead to control of gene expression. As both TCR and chemokine receptor signalling induce LFA-1 adhesion to ICAM-1 (Campbell et al., 1998; Dustin and Springer, 1989; Lloyd et al., 1996) it was of interest to examine the contribution of changes in $[Ca^{2+}]_i$ to the activation of LFA-1.

5.2 RESULTS

5.2.1 Agents that mobilise intracellular Ca^{2+} induce LFA-1 mediated T cell adhesion to ICAM-1

Ionomycin and thapsigargin are agents which can be used to directly raise $[Ca^{2+}]_i$, bypassing the initial signalling events. Ionomycin is an ionophore which complexes with Ca^{2+} and directly transports it out of the ER, whereas thapsigargin inhibits the 100 kDa isoform of the ATP-dependent Ca^{2+} pumps that transport Ca^{2+} from the cytosol into the ER (Papp et al., 1993). Both compounds cause emptying of the intracellular Ca^{2+} stores and a resultant Ca^{2+} influx across the plasma membrane via capacitative entry. To gain insight into the role of intracellular Ca^{2+} in the signalling pathways that activate LFA-1, these two compounds were tested for their ability to activate T cell adhesion to ICAM-1. Ionomycin and thapsigargin both stimulated LFA-1 mediated T cell adhesion to immobilised ICAM-1 in a dose dependent manner (Fig. 5.1). Peak adhesion was achieved with approximately 1 μ M ionomycin and 5 μ M thapsigargin. These results demonstrate a role for intracellular Ca^{2+} in the activation of LFA-1 mediated T cell adhesion to ICAM-1.

5.2.2 An influx of extracellular Ca^{2+} is required for LFA-1 mediated T cell adhesion induced by intracellular Ca^{2+} mobilisation

Ionomycin and thapsigargin raise $[Ca^{2+}]_i$ by capacitative entry across the plasma membrane as well as by inducing release from intracellular stores. The compound SKF-96365, which inhibits capacitative Ca^{2+} entry (Chung et al., 1994),

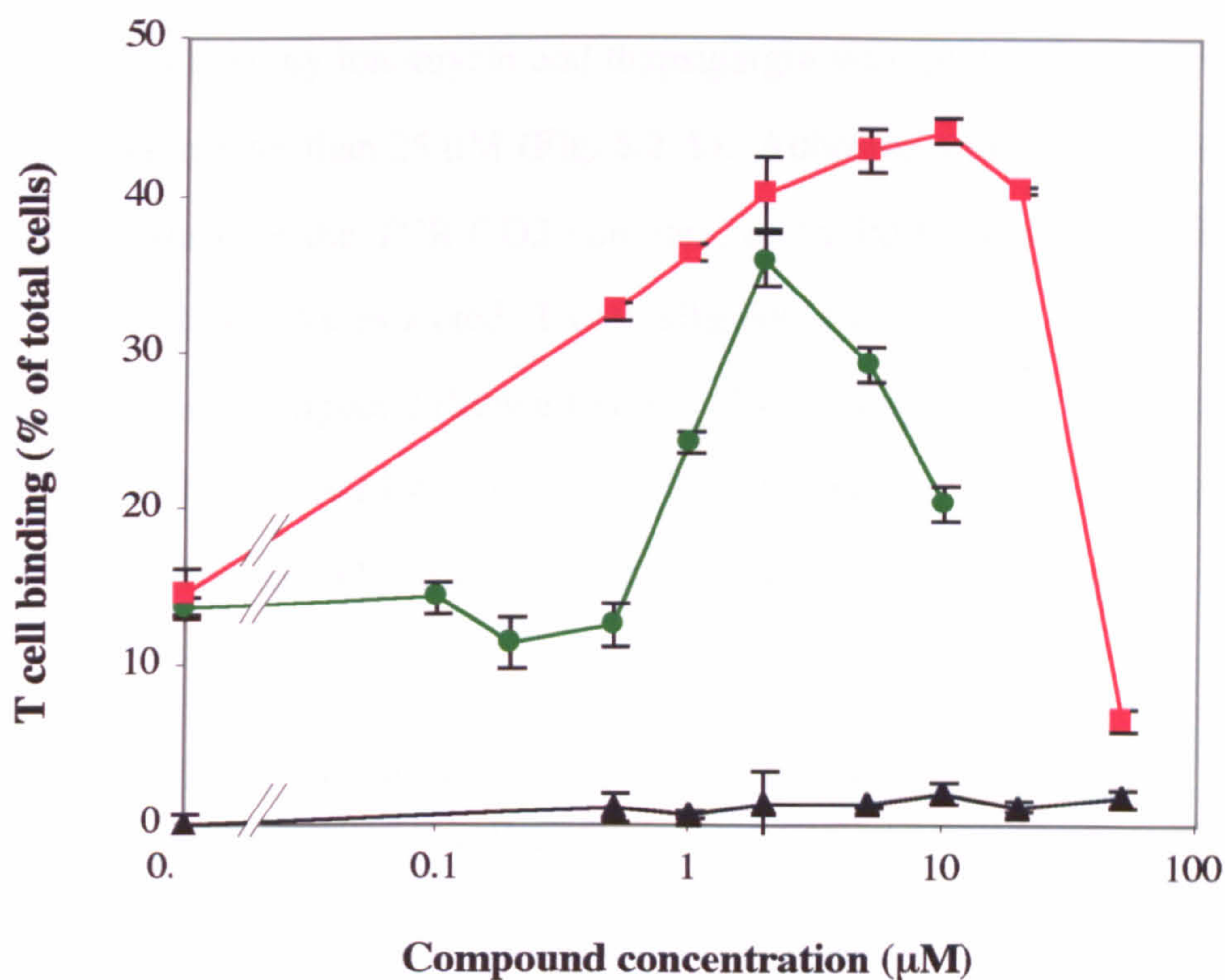


Figure 5.1 The intracellular Ca^{2+} mobilisers ionomycin and thapsigargin induce LFA-1 mediated T cell adhesion to ICAM-1

Stimulation of T cell binding to ICAM-1 with 0-10 μM ionomycin (●) or 0-50 μM thapsigargin (■) for 30 min at 37°C. Thapsigargin stimulated adhesion is inhibited with 5 $\mu\text{g/ml}$ LFA-1 mAb 38 (▲). Data (mean of triplicates \pm S.D.) from one representative experiment (n=2) are shown.

was used to determine whether the initial rise in $[Ca^{2+}]_i$, which results from release from intracellular stores, is sufficient for activation of LFA-1 by inside-out signalling, or whether Ca^{2+} influx across the plasma membrane is also necessary. Adhesion stimulated by ionomycin and thapsigargin was inhibited by SKF-96365 at concentrations greater than 25 μ M (Fig. 5.2 A). Adhesion stimulated by ionomycin, thapsigargin, through the TCR-CD3 complex, or by PdBu was inhibited by SKF-96365 (Fig. 5.2 B). As expected, T cell adhesion stimulated by Mg^{2+} /EGTA was unaffected by this compound (Stewart et al., 1996), even at a concentration of 100 μ M (Fig. 5.2 B). Thus LFA-1 mediated T cell adhesion stimulated by different intracellular signalling pathways requires an influx of extracellular Ca^{2+} .

5.2.3 Expression of the epitope for mAb 24 is low on ionomycin or thapsigargin treated T cells.

The characteristics of ionomycin and thapsigargin stimulated LFA-1 were investigated with the use of mAb 24. Expression of the epitope recognised by this mAb correlates with the high affinity LFA-1 binding to ICAM-1, which occurs upon Mg^{2+} /EGTA stimulation but not upon PdBu stimulation, and is therefore thought to reflect generation of the high affinity conformation of LFA-1 (Stewart et al., 1996). The level of binding of 24 to T cells treated with the Ca^{2+} mobilising agents was similar to that induced by PdBu and very low in comparison to Mg^{2+} /EGTA stimulated binding (Fig. 5.3). It can be concluded that ionomycin and thapsigargin resemble the PdBu model of adhesion by being poor inducers of the high affinity conformation of LFA-1.

5.2.4 Thapsigargin stimulates redistribution of LFA-1 into clusters.

The low binding of mAb 24 indicated that the mechanism of ionomycin and thapsigargin stimulated T cell adhesion to ICAM-1 was unlikely to be via

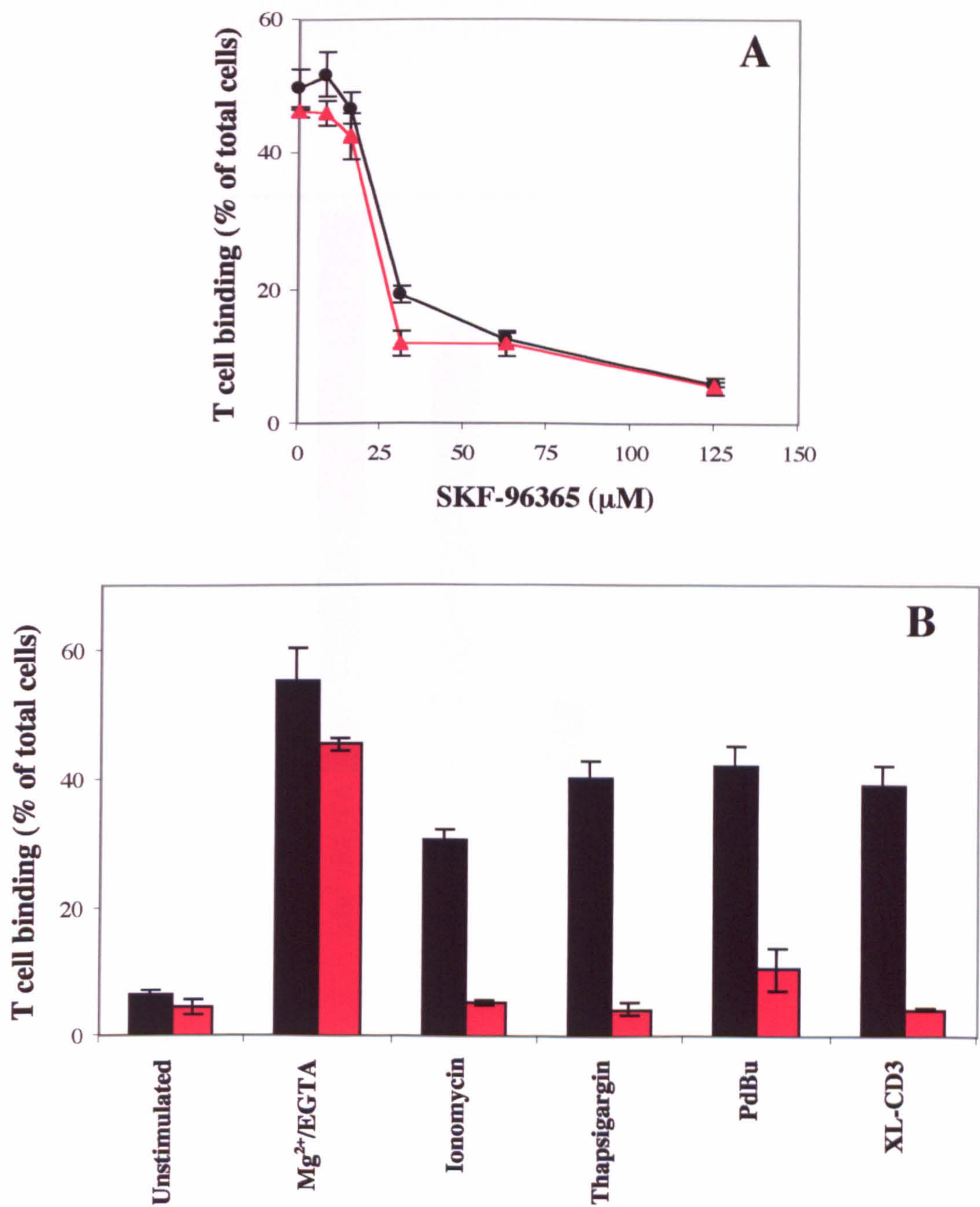


Figure 5.2 Titration of SKF-96365 and its effect on T cell adhesion to ICAM-1
The effect on binding to ICAM-1Fc of preincubation of T cells for 30 min at 37°C **A:** with 0-125 μM SKF-96365 followed by stimulation with 0.7 μM ionomycin (●) or 5 μM thapsigargin (▲) for 30 min at 37°C. **B:** with (■) or without (■) 100 μM SKF-96365 followed by stimulation with 5 mM Mg²⁺/1 mM EGTA, 0.7 μM ionomycin, 5 μM thapsigargin, 50 nM PdBu or 5 μg/ml anti CD3 for 30 min at 37°C. Data (mean of triplicates ± S.D.) from one representative experiment (n=3) are shown.

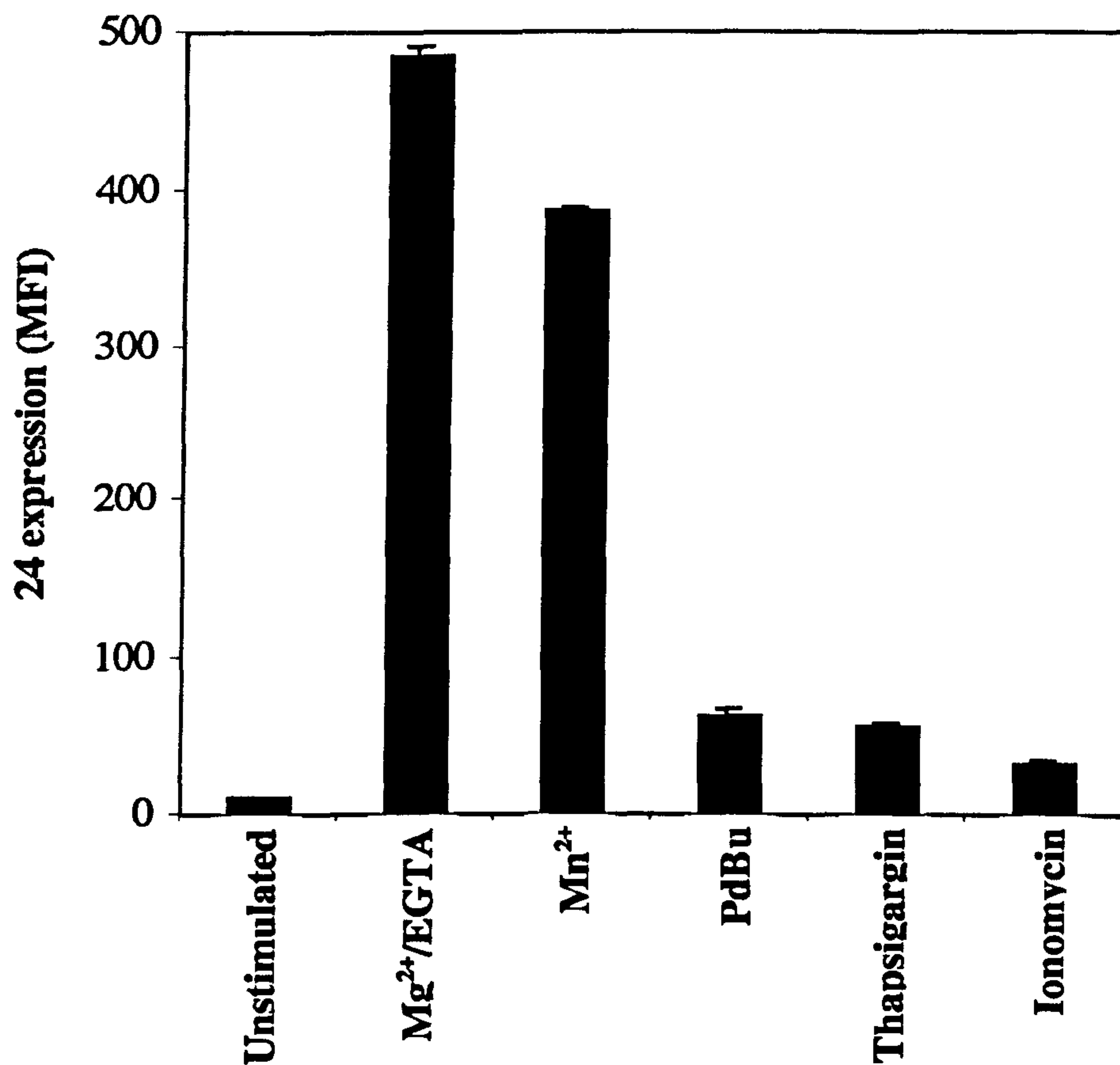


Figure 5.3 The expression of the mAb 24 epitope on T cells following activation of LFA-1

T cells incubated with mAb 24 at 10 $\mu\text{g/ml}$ for 30 min at 37°C in the presence of 5 mM Mg²⁺/1 mM EGTA, 50 μM Mn²⁺, 50 nM PdBu, 5 μM thapsigargin or 0.7 μM ionomycin. Data (mean of triplicates \pm S.D.) from one representative experiment (n=3) are shown.

conversion to a higher affinity form of LFA-1. An alternative mechanism was that the membrane distribution of LFA-1 was altered in a manner facilitating adhesion, therefore confocal microscopy was used to investigate this possibility. In order to analyse LFA-1 distribution, the confocal images were highlighted such that the membrane regions with the highest LFA-1 fluorescence (i.e. in the pixel intensity range 150-250, where 250 is the highest possible value) are depicted in red and all others in white (**Fig. 5.4**). Unstimulated cells (**Fig. 5.4 A**) and Mg^{2+} /EGTA stimulated cells (**Fig. 5.4 B**) have very little high intensity LFA-1 fluorescence (red) in comparison to that on thapsigargin stimulated cells (**Fig. 5.4 C**). These observations were confirmed by quantifying (see **Chapter 2.2.13**) the levels of fluorescence for each sample on regions of the membrane where there was no contact between cells (**Table 5.1: Free Membrane**). Statistical analysis (one way ANOVA) of these measurements confirmed that the level of fluorescence on the thapsigargin stimulated cells, but not on the Mg^{2+} /EGTA stimulated T cells, was significantly increased compared to the level on the resting T cells (**Table 5.1: Significance Levels**). This increase in the intensity of LFA-1 fluorescence upon thapsigargin stimulation was detected with three distinct CD11a mAbs, 38, F110.22 and G25.2 (data not shown), therefore does not represent the exposure of a particular epitope. These results suggested that thapsigargin might stimulate an increase in the cell surface expression of LFA-1. When measured by flow cytometry, however, results revealed that the level of LFA-1 expression following thapsigargin stimulation did not differ from LFA-1 expression on the resting or Mg^{2+} /EGTA treated cells (**Fig. 5.5**). This suggested that the increase in signal strength observed by confocal microscopy upon thapsigargin stimulation reflects increased clustering which creates higher LFA-1 fluorescence intensity.

It was observed that some of the brightest LFA-1 fluorescence was to be found where cells were in contact with each other and, therefore, the level of fluorescence in these regions was quantified (**Table 5.1: Contact Zone**) to determine

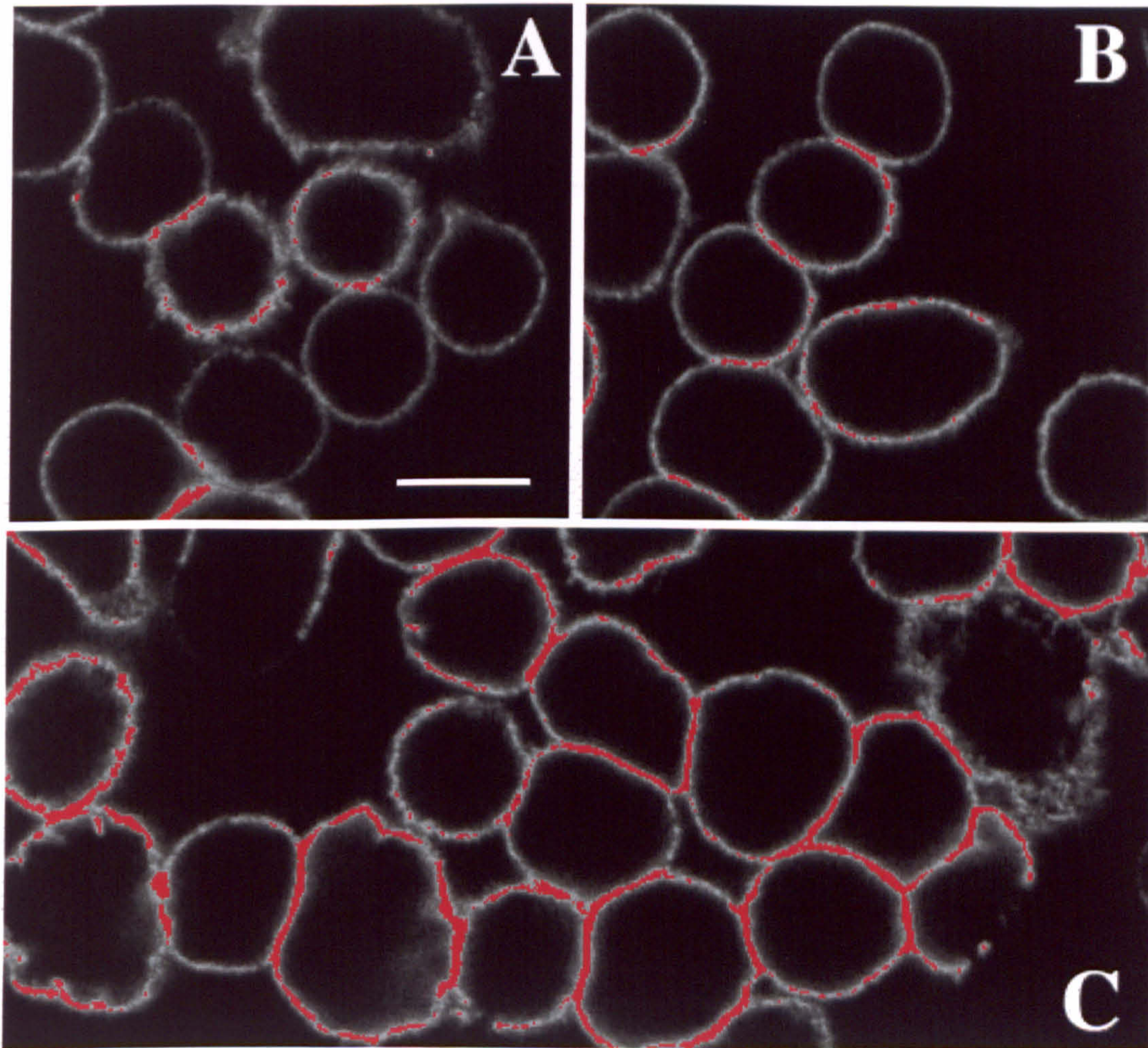


Figure 5.4 Thapsigargin stimulates LFA-1 clustering on T cells

Confocal microscopy images showing the expression of LFA-1 on T cells which were **A:** unstimulated or treated with **B:** 5 mM Mg²⁺/1 mM EGTA or **C:** 5 μM thapsigargin for 30 min at 37°C. Fluorescence intensity exceeding a preset pixel value (see **Chapter 2.2.12**) is coloured red. This setting remained constant in all samples. Bar represents 10 μm.

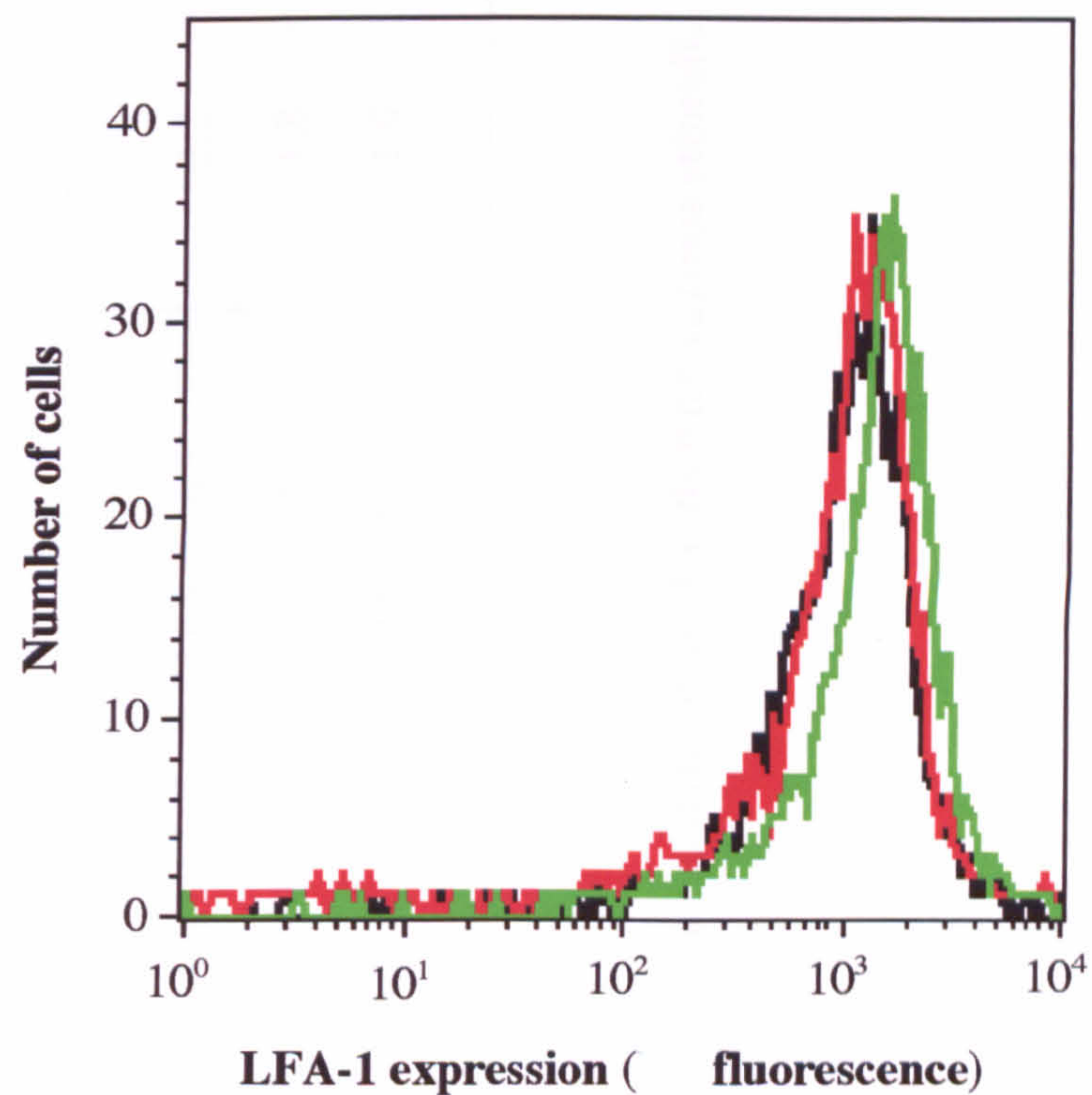


Figure 5.5 The effect of thapsigargin on LFA-1 expression on T cells

The expression of LFA-1 detected by mAb 38 (10 μg/ml) on unstimulated T cells (—) or on T cells stimulated with 5 mM Mg²⁺/1 mM EGTA (—) or 5 μM thapsigargin (—) for 30 min at 37°C. Data from one representative experiment (n=3) are shown.

	Free Membrane (fluorescence units \pm SEM)	Significance Levels (compared to resting T cells) <i>F</i> 1,103 <i>P</i>	Contact Zone (fluorescence units \pm SEM)	Contact/Free
Unstimulated	58.2 \pm 6.1 n=23	-	112.4 \pm 10.4	1.9
Mg ²⁺ /EGTA	60.5 \pm 4.8 n=33	0.09 > 0.1	131.8 \pm 6.5	2.2
Thapsigargin	102.3 \pm 4.8 n=33	31.90 < 0.0001	185.8 \pm 5.7	1.8
Thapsigargin & calpeptin	57.8 \pm 7.6 n=18	0.00 > 0.1	109.6 \pm 14.1	1.9

Table 5.1 Detection of LFA-1 by confocal microscopy following activation of T cells with various stimuli.

whether this high fluorescence reflected a redistribution of LFA-1 to cell:cell contacts or was merely the additive value of the two cell membrane measurements. As the ratio of the average fluorescence intensity at cell contact areas to that of free membrane was approximately 2 (**Table 5.1: Contact/Free**) it can be concluded that there is no large scale redistribution of LFA-1 to points of cell contact upon thapsigargin treatment. In summary, the confocal microscopy results revealed that upon thapsigargin stimulation LFA-1 becomes locally clustered, but does not change its distribution to regions of the cell membrane in contact with other cells. Similar results were obtained with the other Ca^{2+} mobilising agents as well as PdBu (data not shown) and by crosslinking the TCR/CD3 (see **Fig. 5.11**), suggesting that local LFA-1 clustering may be a general feature of several T cell adhesion activating protocols.

5.2.5 Thapsigargin induced clustering of LFA-1 is inhibited by Jasplakinolide.

The implication of the above confocal experiments was that the Ca^{2+} -mobilisers promoted LFA-1 clustering. To assess the role of the cytoskeleton in LFA-1 clustering the cell-permeant peptide jasplakinolide, which is isolated from marine sponge, was tested for its effect on the distribution of LFA-1 on thapsigargin treated cells. Jasplakinolide stabilises actin filaments, prevents actin filament disassembly and, with longer treatment, promotes actin polymerisation (Bubb et al., 1994; Cramer, 1999). As seen in **Figure 5.6**, jasplakinolide diminished the fluorescence levels of LFA-1 detected by confocal microscopy (i.e. there is less fluorescence highlighted in red in **Fig. 5.6 B** than in **Fig. 5.6 A**). Identical levels of LFA-1 expression were detected by flow cytometry before and after jasplakinolide treatment (**Fig. 5.7**), indicating that the inhibitor prevented LFA-1 clustering rather than caused receptor loss. It can therefore be concluded that actin reorganisation is necessary for LFA-1 to become clustered on the cell membrane.

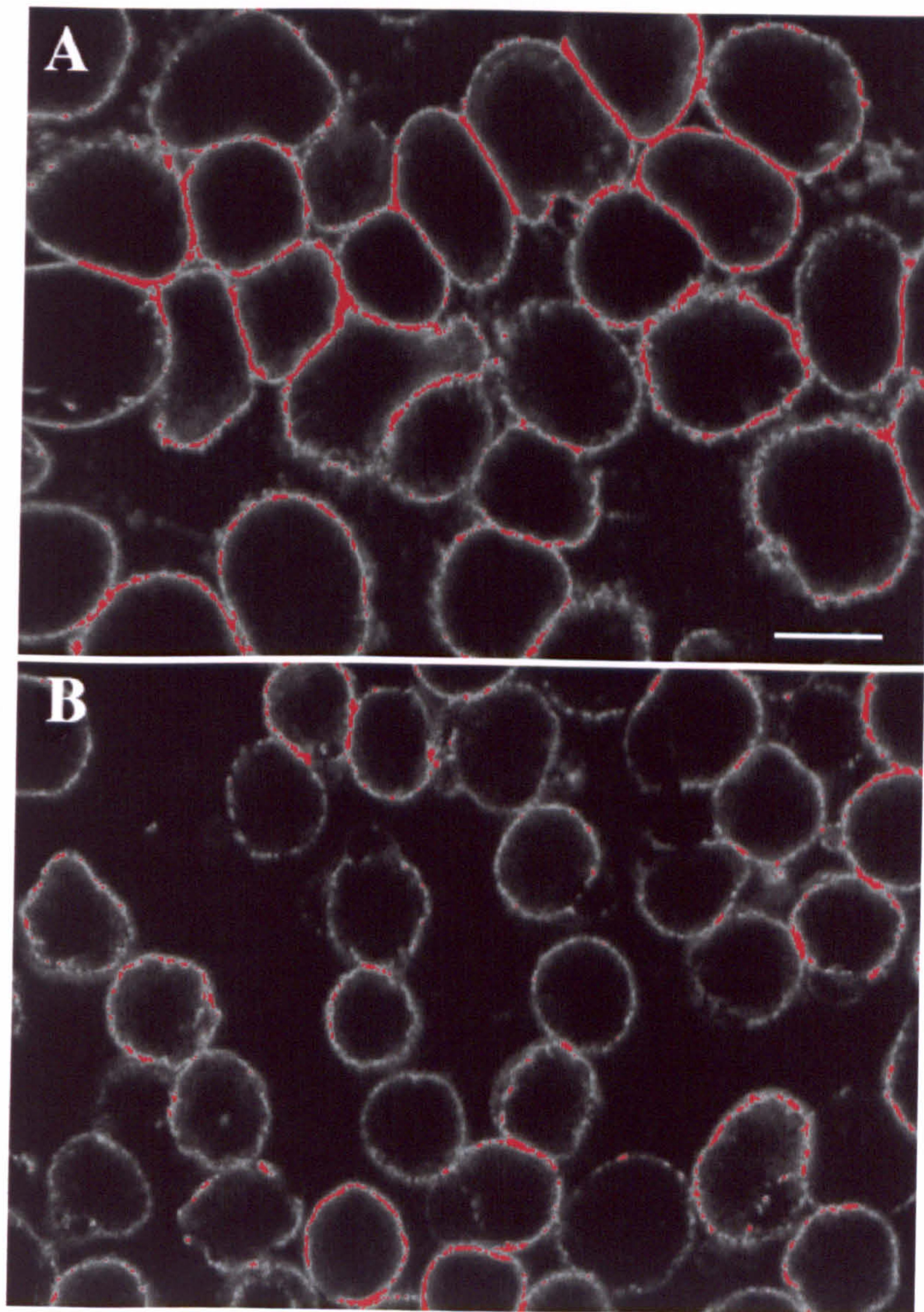


Figure 5.6 The effect of jasplakinolide on thapsigargin induced clustering of LFA-1 on T cells

Confocal microscopy images showing the expression of LFA-1 on T cells which were **A:** treated with 5 μ M thapsigargin for 30 min at 37°C or **B:** preincubated with 1 μ M jasplakinolide for 30 min at 37 °C before treatment as in A. Fluorescence intensity exceeding a preset pixel value (see **Chapter 2.2.12**) is coloured red. This setting remained constant in all samples. Bar represents 10 μ m.

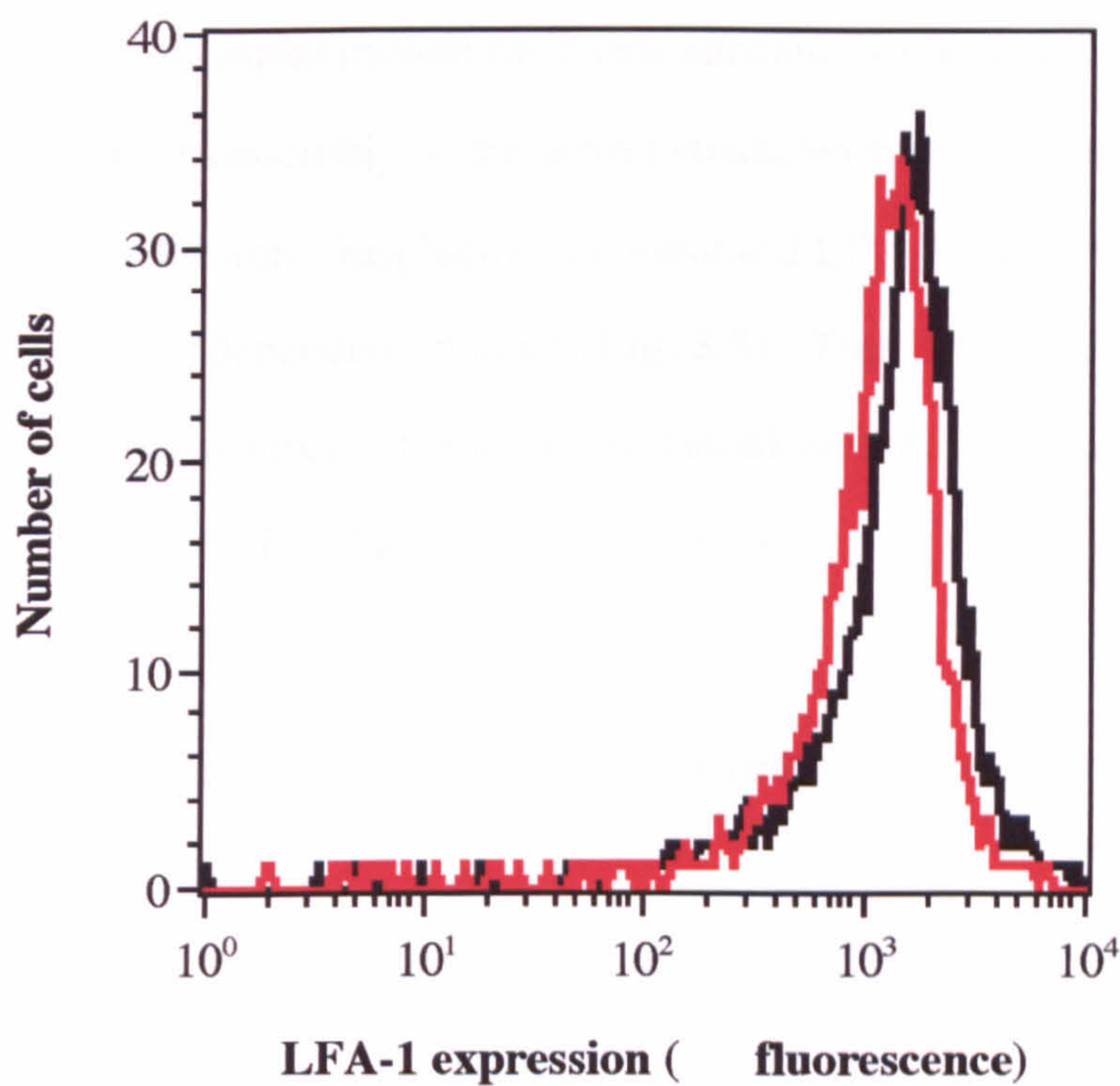


Figure 5.7 The effect of jasplakinolide on LFA-1 expression
The expression of LFA-1 detected with mAb 38 (10 μ g/ml) on T cells incubated at 37°C for 30 min in the presence of 5 μ M thapsigargin (—), or 5 μ M thapsigargin + 1 μ M jasplakinolide (—). Data from one representative experiment (n=3) are shown.

5.2.6 Thapsigargin stimulated T cell adhesion to ICAM-1 is inhibited by Jasplakinolide

The effect of jasplakinolide on T cell adhesion to ICAM-1 was also tested to determine whether disassembly of the actin cytoskeleton and clustering of LFA-1 are requirements for adhesion. Jasplakinolide inhibited LFA-1 mediated T cell adhesion to ICAM-1 in a dose dependent manner (**Fig. 5.8**). Thus LFA-1 clustering, which is dependent on disassembly of the actin cytoskeleton, is a critical feature of thapsigargin stimulated T cell adhesion to ICAM-1.

5.2.7 The calpain inhibitors CBZ-LVG and calpeptin prevent LFA-1 adhesion to ICAM-1

In an attempt to understand the molecular mechanism by which Ca^{2+} induces LFA-1 clustering, the effect of inhibitors and mAbs specific for several major Ca^{2+} -utilising enzymes which have a link with adhesion were investigated in this laboratory (Stewart et al., 1998). Among the inhibitors tested were CBZ-LVG and calpeptin, membrane permeable inhibitors of the cytosolic cysteine protease calpain. Activation of calpain requires intracellular Ca^{2+} levels much higher than those found in resting cells (5 μM to 1 mM depending on the isoform) (Johnson and Guttman, 1997). T cell adhesion to ICAM-1 induced by thapsigargin and PdBu was inhibited by CBZ-LVG in a dose dependent manner, but no effect on Mg^{2+} /EGTA stimulated adhesion was seen (**Fig. 5.9 A**). A variety of stimuli were tested for sensitivity to calpeptin. T cell adhesion stimulated with thapsigargin, PdBu, or through CD3 was inhibited with 100 $\mu\text{g/ml}$ (280 μM) calpeptin but, as with CBZ-LVG, there was no effect on Mg^{2+} /EGTA stimulated adhesion (**Fig. 5.9 B**). There was no alteration in cell viability at concentrations at which these inhibitors were maximally active (data not shown).

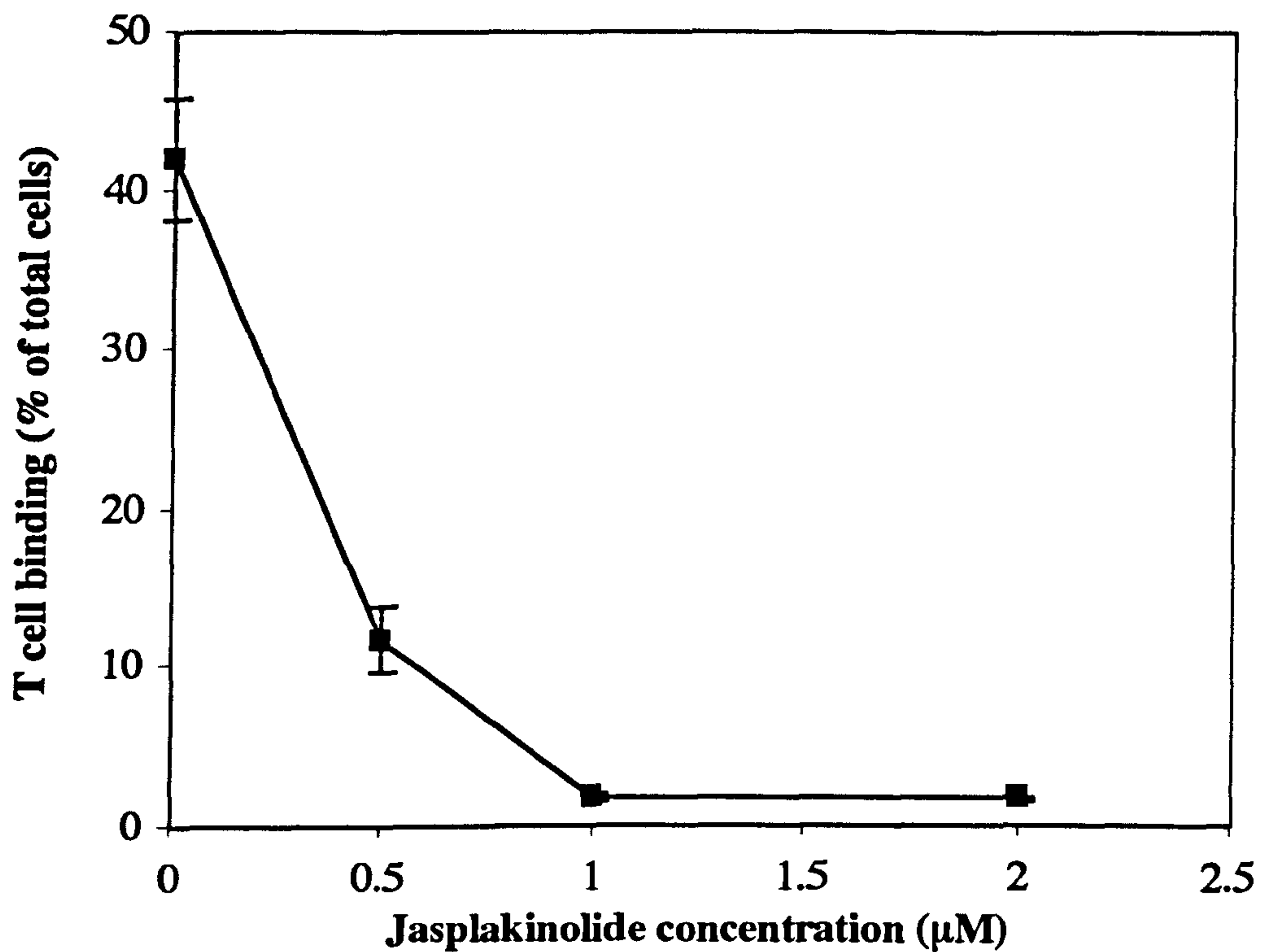


Figure 5.8 The effect of jasplakinolide on LFA-1 mediated T cell adhesion to ICAM-1

The effect on binding to ICAM-1Fc of preincubation of T cells for 1 hour at 37°C with 0-2 μM jasplakinolide followed by stimulation with 5 μM thapsigargin for 30 min at 37°C. Data (mean of triplicates \pm S.D.) from one experiment (n=3) are shown.

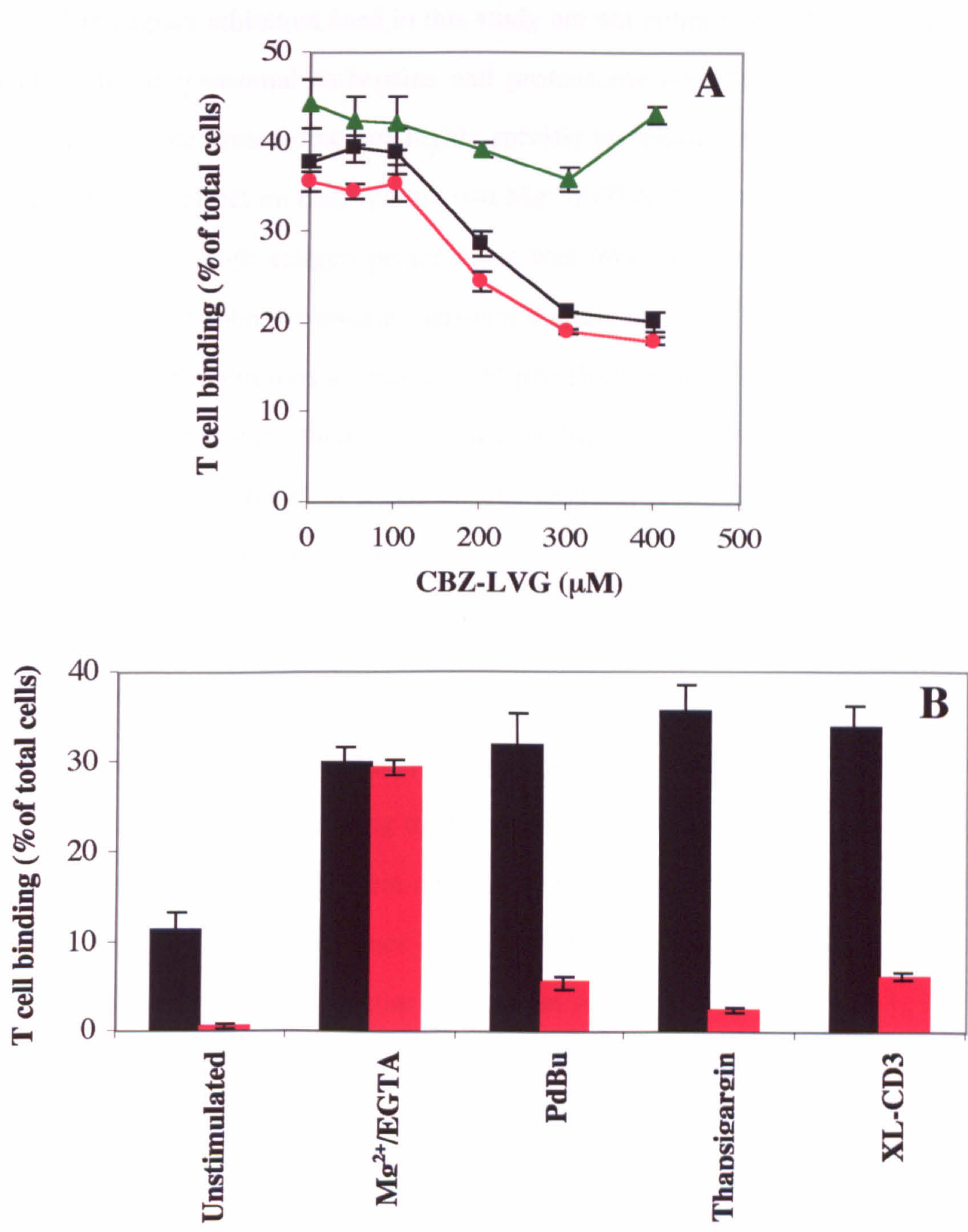


Figure 5.9 The effect of calpain inhibitors on LFA-1 mediated T cell adhesion to ICAM-1

The effect on binding to ICAM-1Fc of preincubation of T cells for 30 min at 37°C **A:** with 0-400 μM CBZ-LVG followed by stimulation with 5 mM Mg^{2+} /1 mM EGTA (\blacktriangle), 50 nM PdBu (\blacksquare) or 5 μM thapsigargin (\bullet) for 30 min at 37°C. **B:** with (\blacksquare) or without (\blacksquare) 280 μM calpeptin followed by stimulation with 5 mM Mg^{2+} /1 mM EGTA, 50 nM PdBu, 5 μM thapsigargin or 5 $\mu\text{g/ml}$ anti-CD3 for 30 min at 37°C. Data (mean of triplicates \pm S.D.) from one representative experiment (n=2 for A; n=4 for B) are shown.

The calpain inhibitors used in this study are not entirely specific for calpain, but also inhibit lysosomal cathepsins and proteasome activity. To rule out the involvement of the proteasome, the highly specific proteasome inhibitor lactacystin was tested for its effect on thapsigargin and Mg^{2+} /EGTA stimulated T cell adhesion to ICAM-1. Although antigen presentation was inhibited by 20 μ M lactacystin (Isabel Correa and John Trowsdale- personal communication) T cell adhesion was unaffected by concentrations as high as 200 μ M (Fig. 5.10). Furthermore, T cells lack lysosomal cathepsins, which are also susceptible to the calpain inhibitors. These results indicate that calpain, or a calpain-like enzyme, is involved in thapsigargin stimulated T cell adhesion to ICAM-1.

5.2.8 Calpeptin inhibits LFA-1 clustering

When examined by confocal microscopy it was found that the levels of clustered LFA-1 detected following treatment with thapsigargin (Fig. 5.11 A) or by crosslinking CD3 (Fig. 5.11 B) were diminished by calpeptin treatment (Fig. 5.11 C and D) (i.e. there is less fluorescence highlighted in red in Fig. 5.11 C and D than in Fig. 5.11 A and B) to levels similar to those on resting T cells (Table 5.1: Free Membrane). Therefore, in conclusion, raising intracellular Ca^{2+} levels in T cells promotes LFA-1 mediated adhesion to ICAM-1 by inducing LFA-1 clustering on the plasma membrane. It would appear that both the clustering and adhesion are dependent upon the actin cytoskeleton and the activity of the Ca^{2+} -dependent protease calpain.

5.2.9 Talin can be cleaved by calpain but does not coprecipitate with LFA-1

Based on these findings it was decided to analyse whether the cytoskeletal associations of LFA-1 alter to allow clustering and ligand binding, with the hope of identifying a calpain dependent step. The following experiments were therefore undertaken to establish which cytoskeletal proteins associate with LFA-1, whether

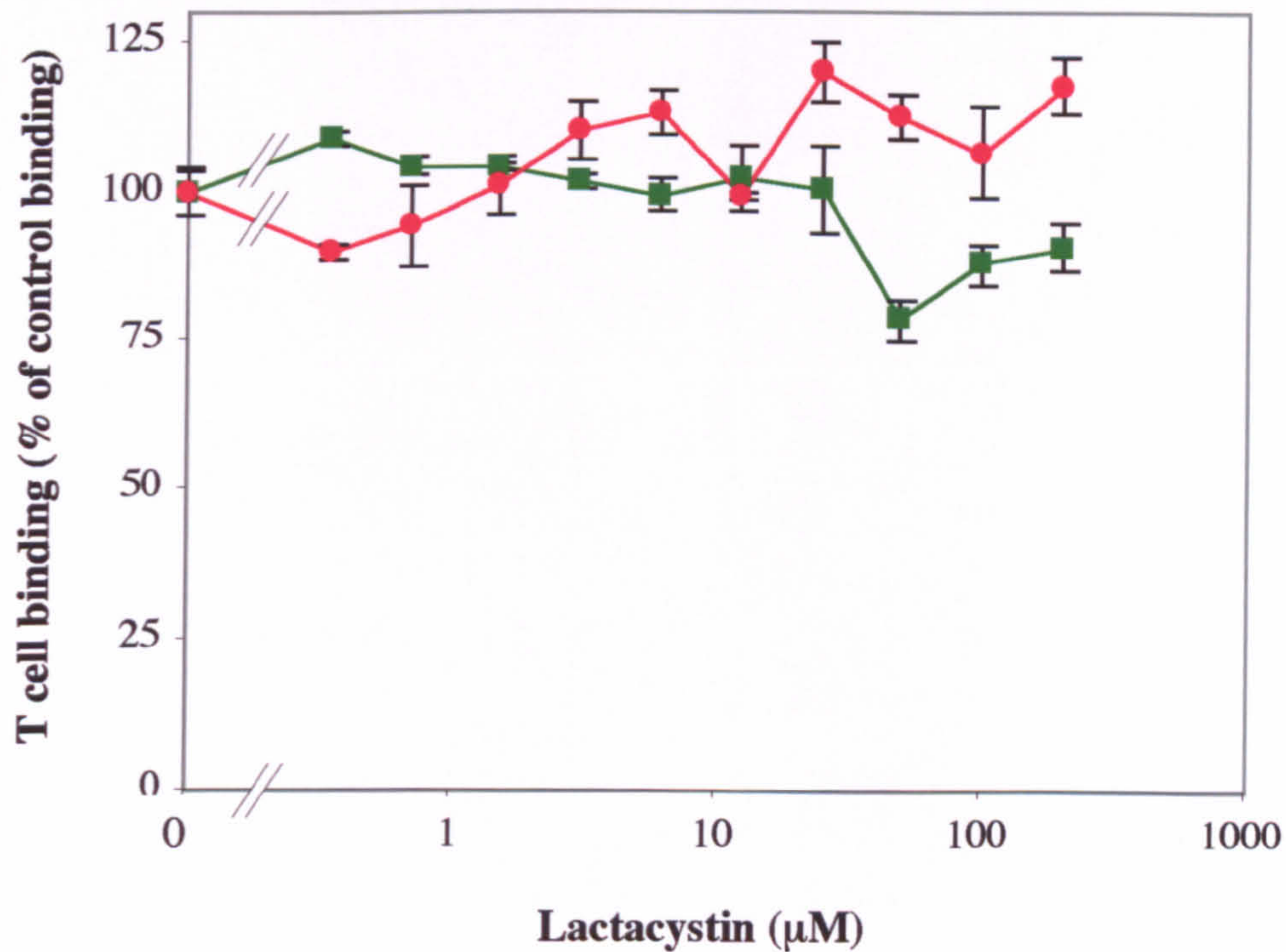


Figure 5.10 The proteasome inhibitor lactacystin has no effect on T cell adhesion to ICAM-1

Binding to ICAM-1 of T cells preincubated for 2 hours at 37°C with 0-200 μM lactacystin then stimulated for 30 min at 37°C with 5 mM Mg²⁺/1 mM EGTA (●) or 5 μM thapsigargin (■). Data from one representative experiment (n=2) are shown.

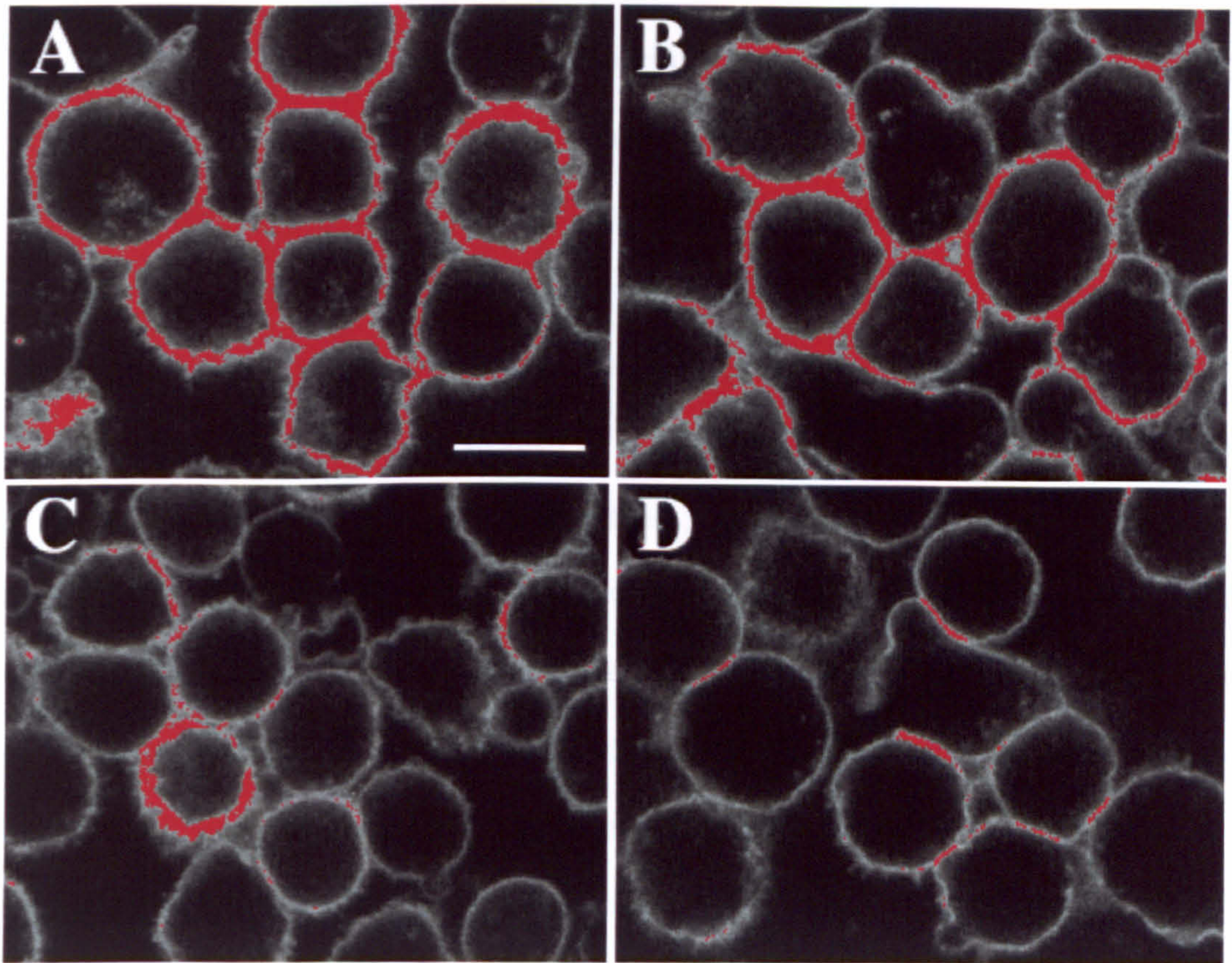


Figure 5.11 LFA-1 clustering induced by thapsigargin and anti-CD3 is inhibited by calpeptin

Confocal microscopy images showing the expression of LFA-1 on T cells which were stimulated with **A and C:** 5 μ M thapsigargin or **B and D:** CD3 mAb G19.4 at 10 μ g/ml for 30 min at 37°C. Cells in **C and D** were preincubated for 30 min at 37°C with 100 μ g/ml calpeptin. Fluorescence intensity exceeding a preset pixel value (see **Chapter 2.2.12**) is coloured red. This setting remained constant in all samples. Bar represents 10 μ m.

these associations are regulated by activation or ligand binding of LFA-1, and whether any of the proteins are cleaved by calpain.

Talin is a 225 kDa cytoskeletal protein that links the plasma membrane and the actin cytoskeleton. Talin co-localises with LFA-1 in the T cell contact zone with an antigen presenting cell (Kupfer and Singer, 1989; Monks et al., 1998). This polarisation apparently occurs in response to LFA-1 engagement by ICAM-1 (Sedwick et al., 1999). Whether talin binds directly to LFA-1 is unclear as opposing results have been obtained from studies of the interaction of purified talin with a β_2 cytoplasmic tail peptide (Sampath et al., 1998; Sharma et al., 1995). Despite this evidence for at least an indirect association of talin with LFA-1, talin was never found to coprecipitate with LFA-1 from resting or activated T cells (Fig. 5.12 A) or upon T cell adhesion to ICAM-1 (data not shown). Calpain cleaves talin into two fragments with apparent masses of 190 kDa and 47 kDa (Rees et al., 1990), however in only 5 out of 15 experiments was a proteolytic fragment of talin detected. On the occasions on which talin was cleaved, this process proved to be activation dependent, occurring in response to T cell stimulation with PdBu, thapsigargin and crosslinking CD3 with mAb UCHT1, but not in unstimulated cells or Mg^{2+} /EGTA treated cells (Fig. 5.12 B). The 190 kDa form of talin was not detected when the cells were pre-incubated with the calpain inhibitor calpeptin (Fig. 5.12 B).

5.2.10 Filamin and α -actinin can coprecipitate with LFA-1 but are not cleaved by calpain

Non-muscle filamin, also known as filamin-1 or actin binding protein 280 (ABP-280), cross-links actin filaments and promotes their high angle branching (Gorlin et al., 1990), as well as mediating the connection of actin with the plasma membrane. There is evidence that filamin binds directly to the integrin β_2 subunit cytoplasmic tail (Sharma et al., 1995). Filamin is a homodimer, each subunit being

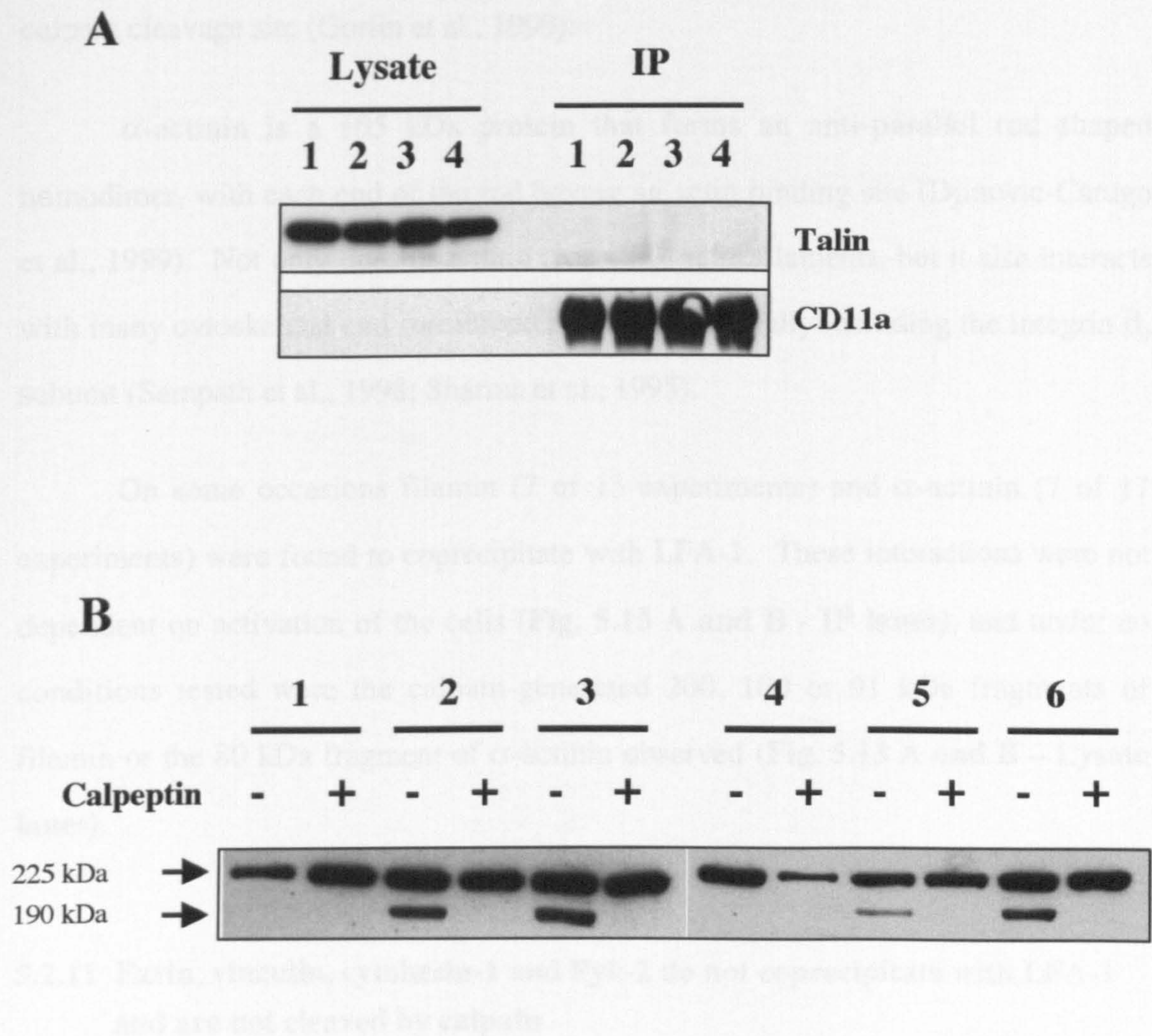


Figure 5.12 Talin does not coprecipitate with LFA-1 but can be cleaved by calpain

A: Talin was detected by western blotting of cell lysates post immunoprecipitation and LFA-1 immunoprecipitates (IP) from unstimulated T cells (lane 1) and T cells stimulated with 2 mM Mg^{2+} /1 mM EGTA (lane 2), 50 nM PdBu (lane 3), or 5 mM thapsigargin (lane 4). In the same samples the LFA-1 α subunit (CD11a) was detected at similar levels in all IPs. **B:** Talin was detected by western blotting of cell lysates from T cells which had (+) or had not (-) been treated for 30 min with 280 μ M calpeptin prior to being unstimulated (lanes 1) or stimulated with 50 nM PdBu (lanes 2), 2.5 μ g/ml UCHT-1 (lanes 3), 2 mM Mg^{2+} /1 mM EGTA (lanes 4), 5 μ M thapsigargin (lanes 5), or 1 μ M ionomycin (lanes 6) for 30 min at 37°C.

280 kDa. The elongated rod-like backbone of filamin is composed of 23 repeat motifs, with a 25 amino acid insertion between repeats 15 and 16 containing the calpain cleavage site (Gorlin et al., 1990).

α -actinin is a 105 kDa protein that forms an anti-parallel rod shaped homodimer, with each end of the rod having an actin binding site (Djinovic-Carugo et al., 1999). Not only does α -actinin cross-link actin filaments, but it also interacts with many cytoskeletal and membrane proteins, potentially including the integrin β_2 subunit (Sampath et al., 1998; Sharma et al., 1995).

On some occasions filamin (7 of 13 experiments) and α -actinin (7 of 17 experiments) were found to coprecipitate with LFA-1. These interactions were not dependent on activation of the cells (Fig. 5.13 A and B - IP lanes), and under no conditions tested were the calpain-generated 200, 100 or 91 kDa fragments of filamin or the 80 kDa fragment of α -actinin observed (Fig. 5.13 A and B - Lysate lanes).

5.2.11 Ezrin, vinculin, cytohesin-1 and Pyk-2 do not coprecipitate with LFA-1 and are not cleaved by calpain

Other proteins associated with cytoskeletal organisation were also tested for interaction with LFA-1 and calpain-dependent cleavage. The ERM family of proteins, ezrin, radixin and moesin, link the plasma membrane to the actin cytoskeleton by binding directly or indirectly to the plasma membrane at their N-terminal and to F-actin at their C-terminal. In lymphocytes stimulated with phorbol ester and ionomycin, ezrin is reported to be cleaved from 80 kDa to 55 kDa by calpain (Shcherbina et al., 1999). Vinculin is a 117 kDa cytoskeletal protein that localises to cell-cell and cell-extracellular matrix contacts. It is composed of a trilobular head connected by a hinge region to a long flexible tail. Binding sites for

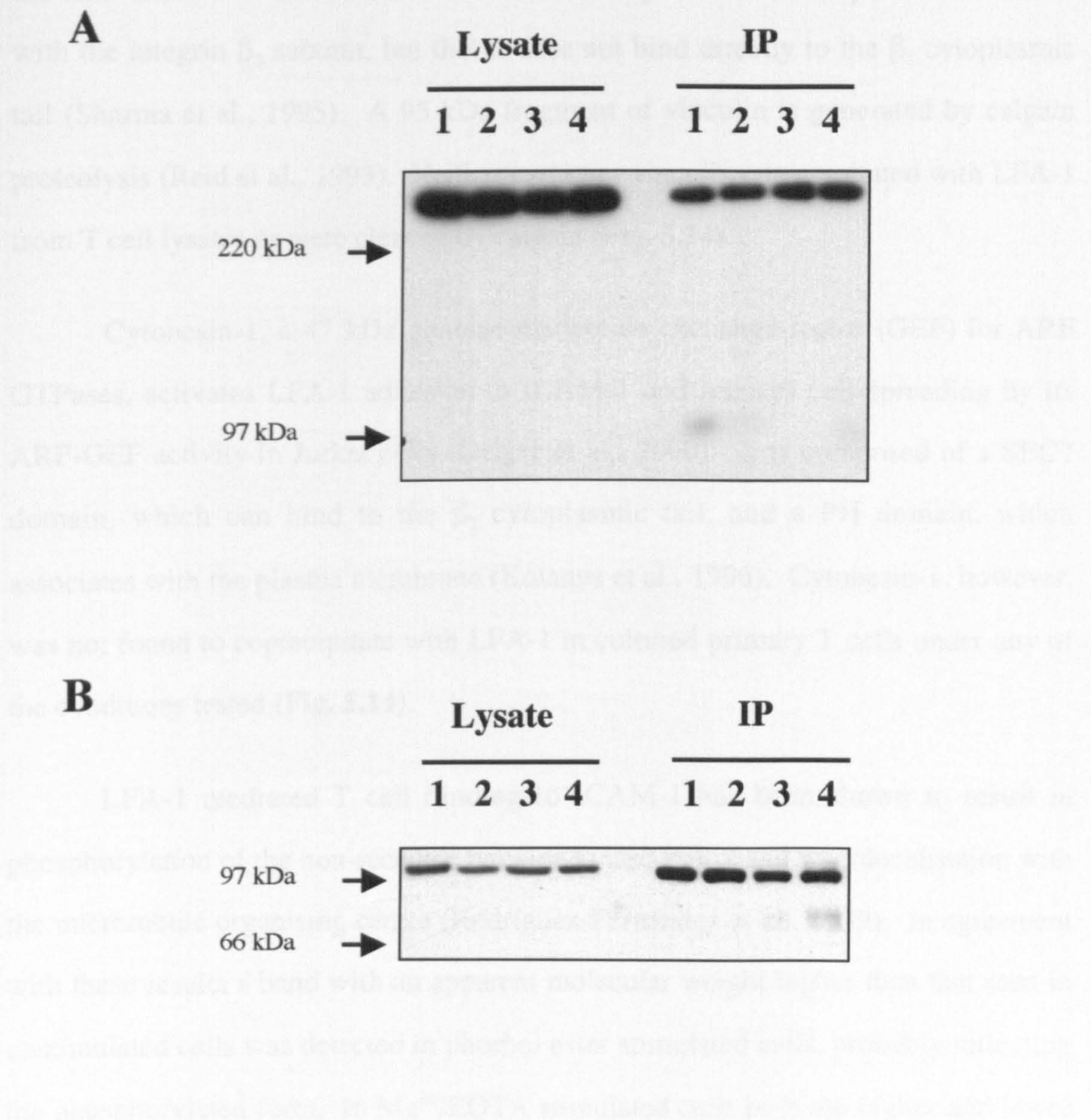


Figure 5.13 Filamin and α -actinin are not cleaved by calpain but can co-precipitate with LFA-1

Western blots of cell lysates and LFA-1 immunoprecipitates (IP) from unstimulated T cells (lane 1) and T cells stimulated with 2 mM Mg^{2+} /1 mM EGTA (lane 2), 50 nM PdBu (lane 3), or 5 mM thapsigargin (lane 4) probed for **A:** filamin and **B:** α -actinin. In the same samples the LFA-1 α subunit (CD11a) was detected at equal levels in all IPs (data not shown). Arrows indicate the position of the molecular weight markers.

talin and α -actinin are located in the head region whereas paxillin and F-actin bind to the tail. There is evidence that vinculin is a component of a complex that interacts with the integrin β_2 subunit, but that it does not bind directly to the β_2 cytoplasmic tail (Sharma et al., 1995). A 95 kDa fragment of vinculin is generated by calpain proteolysis (Reid et al., 1993). Neither ezrin nor vinculin coprecipitated with LFA-1 from T cell lysates or were cleaved by calpain (Fig. 5.14).

Cytohesin-1, a 47 kDa guanine-nucleotide exchange factor (GEF) for ARF GTPases, activates LFA-1 adhesion to ICAM-1 and induces cell spreading by its ARF-GEF activity in Jurkat cells (Geiger et al., 2000). It is composed of a SEC7 domain, which can bind to the β_2 cytoplasmic tail, and a PH domain, which associates with the plasma membrane (Kolanus et al., 1996). Cytohesin-1, however, was not found to coprecipitate with LFA-1 in cultured primary T cells under any of the conditions tested (Fig. 5.14).

LFA-1 mediated T cell binding to ICAM-1 has been shown to result in phosphorylation of the non-receptor tyrosine kinase Pyk-2 and its colocalisation with the microtubule organising centre (Rodríguez-Fernández et al., 1999). In agreement with these results a band with an apparent molecular weight higher than that seen in unstimulated cells was detected in phorbol ester stimulated cells, probably reflecting the phosphorylated form. In Mg^{2+} /EGTA stimulated cells both the higher and lower molecular weight forms of Pyk-2 could be detected (Fig. 5.14). Stimulation of platelets induces rapid phosphorylation of Pyk-2 and upon platelet aggregation Pyk-2 is then cleaved by calpain, from 125 kDa to two fragments of 80 and 75 kDa (Raja et al., 1997). No cleavage of Pyk-2 was detected in T cells (Fig. 5.14).

5.3 DISCUSSION

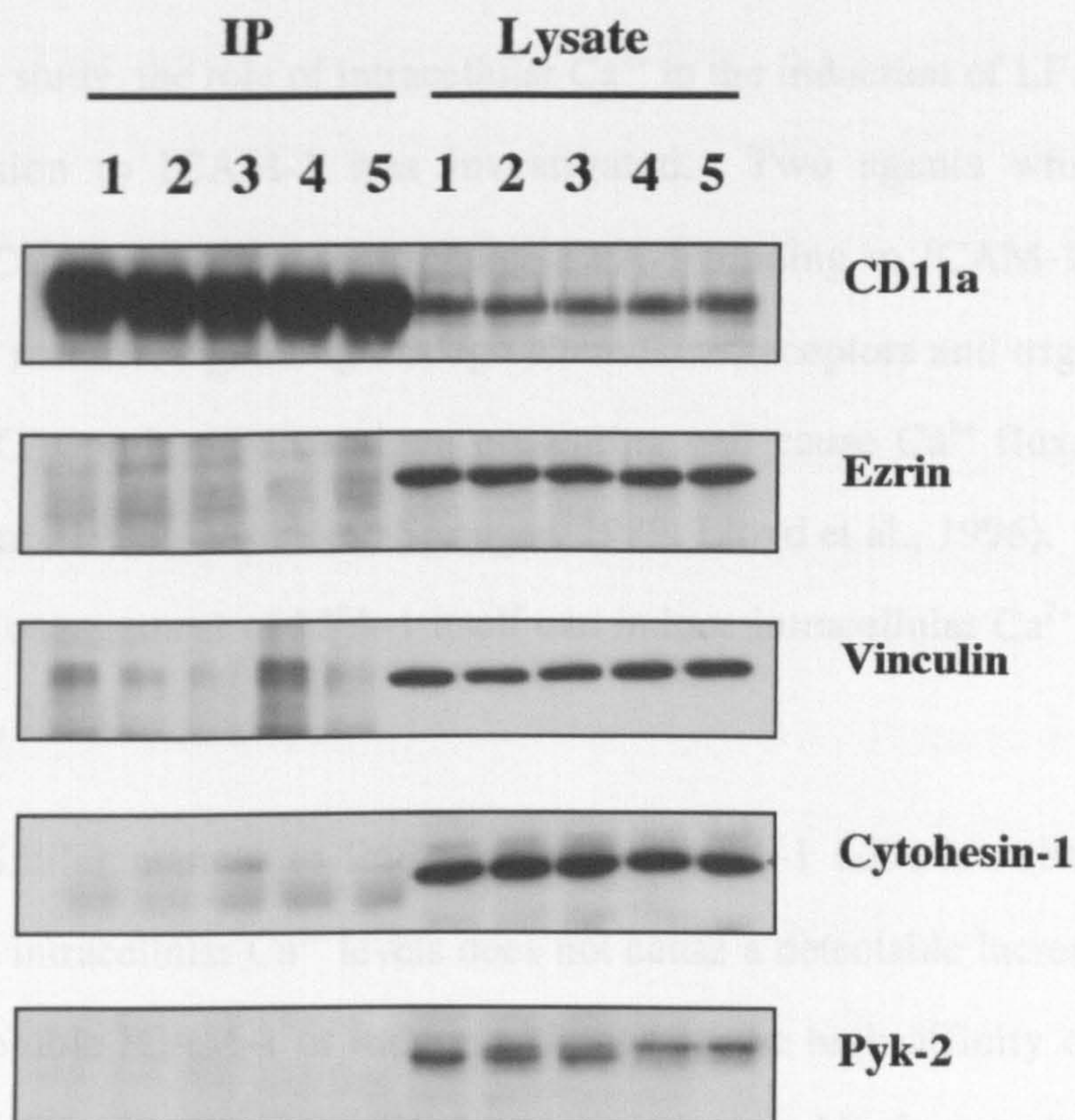


Figure 5.14 Ezrin, vinculin, cytohesin-1 and Pyk-2 do not coprecipitate with LFA-1 and are not cleaved by calpain

Western blots of LFA-1 immunoprecipitates (IP) and cell lysates from unstimulated T cells (lane 1), T cells in suspension stimulated with 2 mM Mg²⁺/1 mM EGTA (lane 2), T cells on ICAM-1 coated plates stimulated by 2 mM Mg²⁺/1 mM EGTA (lane 3), T cells in suspension stimulated with 50 nM PdBu (lane 4), T cells on ICAM-1 coated plates stimulated with 50 nM PdBu (lane 5). All stimulations were performed at 37°C for 30 min. The blots are immunostained with antibodies to the LFA-1 α subunit (CD11a), the cytoskeletal proteins ezrin and vinculin, the guanine-nucleotide exchange factor cytohesin-1 and the non-receptor tyrosine kinase Pyk-2. Representative blots are shown ($n \geq 3$ for each blot).

5.3 DISCUSSION

In this study, the role of intracellular Ca^{2+} in the induction of LFA-1 mediated T cell adhesion to ICAM-1 was investigated. Two agents which mobilise intracellular Ca^{2+} were found to stimulate LFA-1 binding to ICAM-1. In a more physiological manner, signalling through chemokine receptors and triggering of the TCR by MHC/peptide on an antigen presenting cell cause Ca^{2+} fluxes in T cells (Campbell et al., 1998; Dustin and Springer, 1989; Lloyd et al., 1996). There is also evidence that engagement of LFA-1 itself can induce intracellular Ca^{2+} mobilisation (Kanner et al., 1993).

In a similar manner to PdBu stimulated LFA-1 adhesion (Stewart et al., 1998), raising intracellular Ca^{2+} levels does not cause a detectable increase in LFA-1 affinity for soluble ICAM-1 or induce a change to the high affinity conformation recognised by mAb 24. Instead, LFA-1 becomes clustered in the membrane and thus the avidity of interaction with ICAM-1 is increased. In a model of $\alpha_4\beta_1$ adhesion, integrin clustering is driven by ligand (Yauch et al., 1997). It is unlikely that the LFA-1 clusters detected in this study are driven by ligand as they are observed uniformly over the entire cell surface, not only at cell-cell contacts, and can be detected by both non-function blocking and function blocking mAbs (data not shown).

The use of inhibitors implicated calpain in the clustering and adhesion of LFA-1. Calpains are a family of intracellular neutral cysteine proteases that are activated by local Ca^{2+} fluxes and are widely expressed in mammalian cells (reviewed in (Johnson and Guttman, 1997; Sorimachi et al., 1997)). Calpains are highly expressed in T cells and are increased at mRNA and protein levels by phorbol ester, calcium ionophore and anti-CD3 treatments, all agents that can induce LFA-1 adhesion (Deshpande et al., 1995). In platelets, addition of the agonist thrombin

induces a relocation of calpain to the cell periphery where it is subsequently activated by binding of ligand to the integrin $\alpha_{\text{Lb}}\beta_3$ (Fox et al., 1993). LFA-1 engagement can also result in calpain activation (Soede et al., 1999) but, as discussed above, it is unlikely that ligand is participating in the calpain dependent clustering of LFA-1. However, this does not preclude further action of calpain after ICAM-1 binding of T cells.

There are a number of possibilities to explain how calpain could be acting. Calpain may be activated to cleave a key protein, physically releasing LFA-1 from its cytoskeletal restraint and allowing movement in the membrane as has been observed by single particle tracking (Kucik et al., 1996). Proteins that have been identified as calpain targets include talin (Rees et al., 1990), filamin (Collier and Wang, 1982) and α -actinin (Selliah et al., 1996), all of which have been reported to associate with integrins (reviewed in (Critchley, 2000)). In resting neutrophils the integrin β_2 subunit is associated with the intact form of talin, but upon neutrophil activation talin is cleaved by calpain, dissociates from the integrin, and is replaced by α -actinin (Sampath et al., 1998). Whether a similar process occurs in T cells was investigated. Although talin proteolysis by calpain was seen at times, this result was not consistent and at no point was talin seen to coprecipitate with LFA-1. α -actinin did associate with LFA-1 on some occasions, however this interaction was not dependent on the activation state of the cell. These discrepancies with the neutrophil results may reflect a cell specificity to the interactions. For T cells it is upon activation, not in resting conditions, that LFA-1 and talin colocalise in the immunological synapse (Monks et al., 1998) and activation of freshly isolated T cells results in α -actinin, not talin, being cleaved by calpain (Selliah et al., 1996). The cytoskeletal proteins filamin, vinculin and ezrin also did not coprecipitate with LFA-1 or undergo proteolysis by calpain. Another possibility is that calpain cleaves a protein involved in the signalling pathways leading to integrin activation. Cytohesin-1, Pyk-2, p125^{FAK} and protein tyrosine phosphatase 1B are candidates, as all but

cytohesin-1 are known targets of calpain proteolysis (Cooray et al., 1996; Raja et al., 1997; Rock et al., 1997) and all are involved in integrin signalling (Arregui et al., 1998; Kolanus et al., 1996; Lipfert et al., 1992; Rodríguez-Fernández et al., 1999). Some integrin β subunit cytoplasmic tails can be cleaved by calpain (Du et al., 1995; Potts et al., 1994), including the β_2 subunit (Pfaff et al., 1999), however this possibility remains to be investigated.

It should be noted that problems were encountered with the co-precipitation experiments. In attempts to obtain consistent results, lysis buffers were tried containing a range of concentrations of the non-ionic detergents Triton X 100 or Brij 96 (polyoxyethylene 10 oleyl ether) or the zwitterionic detergent CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate). The lysis buffer was also varied to include $MgCl_2$ and sucrose to stabilise F-actin. The cells were allowed to adhere to ICAM-1 to discover whether this would stabilise interactions, and also various immunoprecipitating antibodies tested and added before cell lysis or to the precleared lysate. None of these conditions improved the reproducibility of the results (data not shown). The coprecipitation results should therefore be viewed with scepticism. Experiments are required to verify that when coprecipitation of α -actinin and filamin with LFA-1 is seen it is real and not just non-specific binding to the sepharose beads or antibody. The lysates were precleared to minimise this possibility and the immunoprecipitates thoroughly washed in the presence of salt. However, it remains a possibility, until controls are performed, that α -actinin and filamin are stickier than the other proteins tested, or that they are in a sticky complex that binds to the beads.

Other studies have also demonstrated a role for calpain in integrin-mediated events. Cell motility is mediated by integrins and can be dissected into four phases: extension of lamellipodia, formation of new focal adhesions at the leading edge, detachment at the trailing edge and translocation of the cell mass. Failure of any of these processes prevents motility. Calpain has a vital role in cell migration, probably

by facilitating constant reorganisation of focal adhesions and other cell-substratum contacts that are required for motility. Inhibition of calpain can prevent fibroblast spreading and actin cytoskeletal reorganisation (Potter et al., 1998), and this is thought to reflect a role for calpain upstream of Rac and Rho (Kulkarni et al., 1999). Motile fibroblasts in which calpain is inhibited form stable focal adhesions and cannot break the contacts at the rear of the cell, resulting in restrained motility (Huttenlocher et al., 1997). Recently, it has been shown that fibroblast de-adhesion and motility induced by epidermal growth factor (EGF) occurs as a result of m-calpain activation downstream of ERK/MAP kinase signalling (Glading et al., 2000). Increases in intracellular cAMP levels, for instance upon chemokine binding to CXCR3, prevent this EGF stimulated motility by preventing m-calpain activation in a protein kinase A dependent manner (Shiraha et al., 1999). It is interesting that in fibroblasts calpain inhibitors are not reported to decrease the number of adherent cells. This is in contrast to the results with T cells presented here. This apparent discrepancy requires further examination: are there different roles for calpain in different cell types, or does calpain inhibition reduce the number of cells that adhere and prevent migration of those cells that do adhere, no matter the cell type?

Many of these results have been demonstrated using calpeptin, one of the more soluble and membrane permeable calpain inhibitors available, but have also been supported by more specific means such as dominant negative calpain mutants, calpain antisense oligonucleotides and by overexpression of the natural calpain inhibitor calpastatin. It remains a possibility that the effect of calpeptin on T cell adhesion to ICAM-1 is not due to inhibition of calpain. Primary T cells, however, are difficult to transfect and microinject, therefore these methods of introducing the more specific inhibitors of calpain into the cell were not attempted. Instead a calpastatin-antennapedia peptide was synthesised. This peptide consisted of a 27 amino acid sequence associated with the inhibitory activity of the endogenous calpain inhibitor protein, calpastatin (Maki et al., 1989) attached to a 16 amino acid

peptide from the third helix of the Antennapedia homeodomain protein to act as an internalisation vector (Hall et al., 1996). This peptide had no effect on T cell adhesion to ICAM-1 (data not shown) and was not further pursued. No conclusion can be drawn from this result without proving that the peptide entered the cell.

Caution should be employed in interpreting results obtained with inhibitors when no other means of substantiating the results is used. For example, calpain inhibitors may also inhibit proteasome activity, however the possibility that this was the case was dismissed by demonstrating that a specific proteasome inhibitor had no effect. Recently, it has been suggested that calpeptin can inhibit a membrane associated tyrosine phosphatase upstream of Rho (Schoenwaelder and Burridge, 1999). In these experiments calpeptin treatment, rather than inhibiting actin remodelling, lead to Rho mediated stress fibre formation. It would be interesting to test whether activation of Rho can be detected upon calpeptin treatment of T cells to rule out the possibility that we are looking at protein tyrosine phosphatase activity. However, our results require intracellular Ca^{2+} whereas Schoenwaelder *et al.* can chelate Ca^{2+} without any effect in their model. In addition, our laboratory has been unable to see any effect of C3 exoenzyme, a Rho inhibitor, on T cell adhesion to ICAM-1 (Mairi Stewart and Jo Porter – unpublished results).

Recently, Patterson *et al.* have shown that treatment of smooth muscle cell lines with 3 μM jasplakinolide for one hour causes redistribution of F-actin into a tight layer around the plasma membrane and that this prevents the coupling of the ER and plasma membrane Ca^{2+} channels which they show is necessary for capacitative Ca^{2+} entry in these cells (Patterson et al., 1999). Although the concentration of jasplakinolide used in **Figure 5.6** and **Figure 5.8** is reported not to disrupt actin-cytoskeleton organisation (Cramer, 1999), the ability of the T cells to flux Ca^{2+} following treatment with jasplakinolide should be tested to establish that the jasplakinolide effects are not due to inhibition of the Ca^{2+} flux. Calpeptin should also

be tested in this way to ensure that it inhibits events downstream of the rise in $[Ca^{2+}]_i$ and is not having its effect on adhesion by preventing the rise in $[Ca^{2+}]_i$.

In summary, this work shows that Ca^{2+} regulated activation of calpain (or a calpeptin sensitive protein) causes clustering of LFA-1. When T cells contact antigen presenting cells (APC), the T cells initially migrate on the APC, but convert to being stably adherent upon Ca^{2+} flux (Negulescu et al., 1996), and LFA-1 is localised to the T cell-APC contact (Monks et al., 1998). The work described in this chapter would fit in with the prediction that an initial Ca^{2+} flux, initiated by T cell contact with an APC, activates calpain. This would allow local clustering of LFA-1, possibly by cleaving an as yet unidentified cytoskeletal or signalling protein. It will be interesting to test whether, as in fibroblasts, calpain also has a role in LFA-1-mediated T cell migration.

CHAPTER 6

CHARACTERISATION OF LEUKOCYTES FROM A PATIENT WITH A NOVEL FORM OF INTEGRIN DYSFUNCTION

6.1 INTRODUCTION

Leukocyte Adhesion Deficiency-1 (LAD-1) and Glanzmann's Thrombasthenia are rare, inherited, immune disorders affecting integrin function (reviewed in (Anderson et al., 1994; George et al., 1990; Hogg and Bates, 2000)). Mutation of the gene encoding the integrin β_2 subunit is responsible for LAD-1. The majority of mutations are in the β I-like domain and prevent association with the α subunit. The lack of $\alpha\beta$ heterodimer formation results in deficient transport of the integrin to the cell surface and absence of expression of all the β_2 integrins (Wardlaw et al., 1990). Mutations in the β_2 subunit that allow expression but not function of the β_2 integrins have also been described (Hogg et al., 1999). LAD-1 is characterised by delayed umbilical cord separation, granulocytosis, reduced emigration of phagocytic cells to inflamed tissue, impaired phagocytosis and pus formation, recurrent or progressive infections of soft tissue and susceptibility to recurring, life threatening, bacterial infections. Delayed-type hypersensitivity reactions are normal, there are no problems with viral infections and antibody production is usually present to some extent. Severely affected people often die of infection in childhood or early adulthood. Bone marrow transplantation is the only curative treatment available at present.

Glanzmann's Thrombasthenia is a bleeding disorder caused by mutations in either the α or β subunit of the platelet integrin $\alpha_{\text{Ib}}\beta_3$. As for LAD-1, the majority of mutations in the β subunit occur in the I-like domain. For the α_{Ib} subunit, most of

the mutations are located in the putative β -propeller domain (reviewed in (Hogg and Bates, 2000)). The mutations result in absence of cell surface expression or expression of abnormal integrin that is unable to bind to fibrinogen. Thus the platelets fail to aggregate and form a clot in response to physiological agonists that are released at sites of vascular injury, such as thrombin, ADP and thrombospondin. Glanzmann's patients therefore suffer from irregular bleeding and easy bruising. Occasionally, severe gastrointestinal and intracranial bleeding occurs, which can lead to death. Patients with severe bleeding are given platelet transfusions but eventually become refractory to these.

Patient FM is an 18 month old girl from Malta with clinical features of both LAD-1 and Glanzmann's Thrombasthenia: she has a history of bleeding problems, recurrent bacterial infections, non-healing skin lesions and raised leukocyte numbers. She first presented only 6 hours after birth with cerebral bleeding. She had a sibling who died of a cerebral bleed and has one other sibling who is now 5 or 6 years old and healthy. As far as can be ascertained her parents are not related.

This chapter describes the defects in the function of leukocytes from this patient. The results suggest that she has a novel form of integrin dysfunction in which the β_1 , β_2 and β_3 integrins are expressed on the cell surface at normal levels but cannot be stimulated to bind ligand by intracellular signalling pathways. Due to the small amount of blood that can be obtained from such a young child and her infrequent visits to hospital in this country, some of the results presented in this chapter, particularly those with neutrophils, are preliminary.

6.2 RESULTS

6.2.1 Analysis of Patient FM Leukocyte Subsets

The number of leukocytes obtained from a heparinised sample of Patient FM's blood, after erythrocytes were depleted by dextran sedimentation, was $32 \times 10^6/\text{ml}$, four times the number obtained from an equal volume of blood from an age matched healthy donor (Control). This was accounted for by a two fold increase in the neutrophil numbers and an approximately ten fold increase in the number of peripheral blood mononuclear cells (PBMC) (Table 6.1).

Neutrophils were separated from peripheral blood mononuclear cells (PBMC) on a Percoll gradient and the two fractions analysed for leukocyte populations by flow cytometry. In particular we were interested in discovering whether any specific PBMC population was responsible for the markedly elevated numbers of PBMC or whether all populations were expanded. T cells were identified by staining for CD3. The proportion of T cells in the patient's PBMC fraction was decreased compared to control, but had a normal CD4:CD8 ratio (Table 6.2 and data not shown). The CD14 positive monocyte population formed a similar proportion of the PBMCs in the patient and control. Of note, the CD19 positive B cell population formed 20% of the patient's PBMCs but only 6% of the control's PBMCs, and the patient also had an increased proportion of CD16 positive PBMCs, probably NK cells (Table 6.2).

6.2.2 Patient FM has normal levels of β_2 integrin expression

Because the patient suffered from symptoms indicative of LAD-1, the cell surface expression of the β_2 integrins was investigated by flow cytometry. As can be seen by the overlapping profiles in Figure 6.1, normal levels of LFA-1, Mac-1 and p150,95 were detected on T cells from Patient FM. The expression of α_x and β_2 on Patient FM's neutrophils were similar to the control levels. Although it appears from

	CONTROL	PATIENT FM
Total leukocytes	8 x 10 ⁶ /ml	32 x 10 ⁶ /ml
PBMC	1.1 x 10 ⁶ /ml	15 x 10 ⁶ /ml
Granulocytes	6 x 10 ⁶ /ml	12 x 10 ⁶ /ml

Table 6.1 Control and Patient FM Leukocyte numbers
The number of leukocytes, peripheral blood mononuclear cells (PBMC) and granulocytes found per ml of blood from Patient FM and a control donor (n=2).

	CONTROL	PATIENT FM
CD3	64	35
CD14	14	19
CD19	6	20
CD16	12	25

Table 6.2 Control and Patient FM PBMC subsets
The percentage of control and Patient FM PBMCs expressing CD3 (UCHT1), CD14 (UCHM1), CD19 (BU12) and CD16 (3G8). Representative results are shown (n=2).

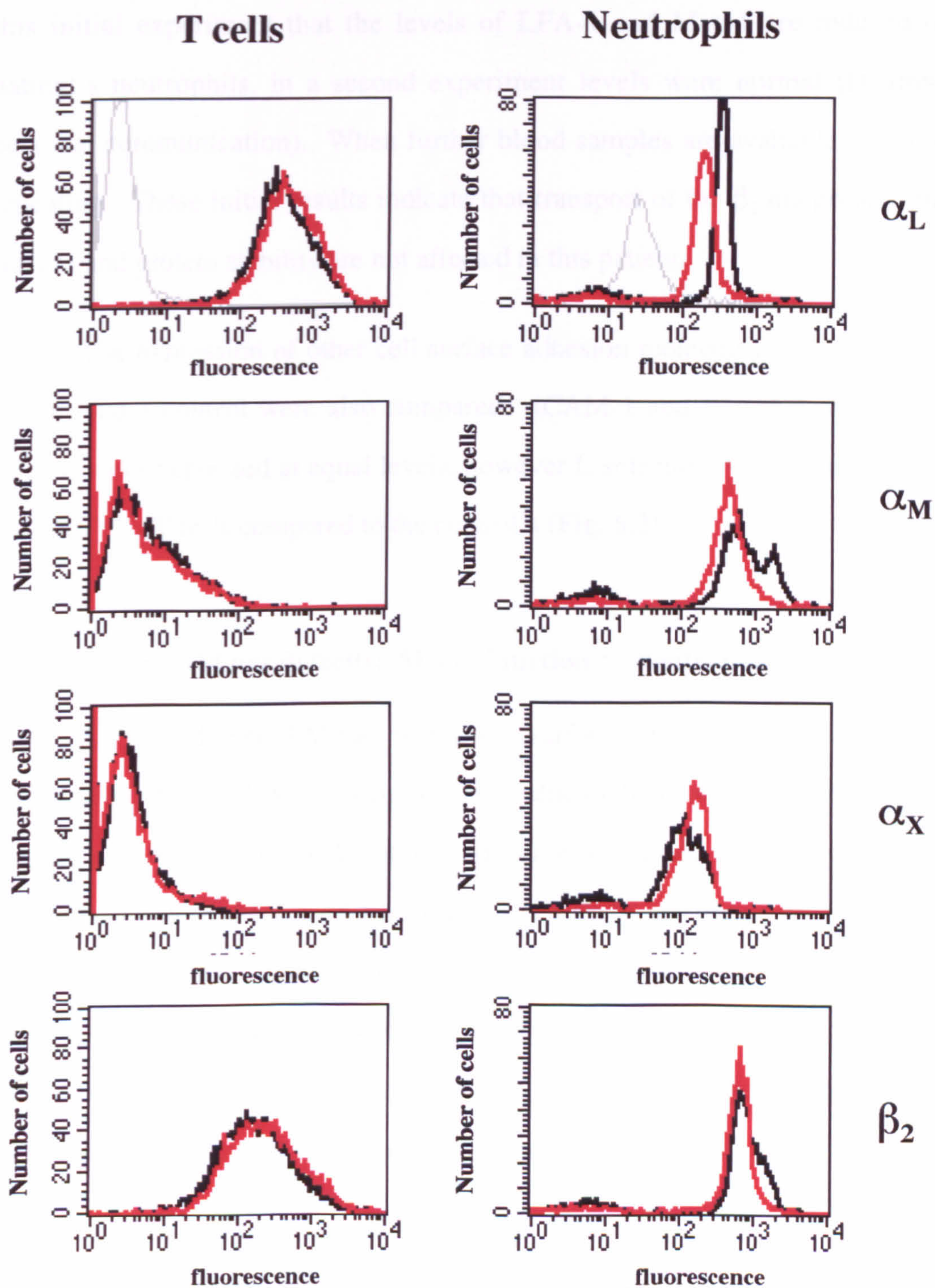


Figure 6.1 Expression of the β_2 integrins on T cells and neutrophils

Expression of α_L (mAb 38), α_M (ICRF44), α_X (3.9) and β_2 (TS1/18) on control (—) and Patient FM (—) T cells and neutrophils. The negative control is indicated (—). Data from one experiment are shown (n=2 for T cells; n=1 for neutrophils).

this initial experiment that the levels of LFA-1 and Mac-1 are reduced on the patient's neutrophils, in a second experiment levels were normal (D. Imwold - personal communication). When further blood samples are available, this requires repeating. These initial results indicate that transport of the β_2 integrins to the cell surface and protein stability are not affected in this patient.

The expression of other cell surface adhesion molecules on T cells from the patient and a control were also compared. ICAM-1 and ICAM-3, two ligands of LFA-1, were expressed at equal levels, however L-selectin levels were decreased on Patient FM's T cells compared to the control's (Fig. 6.2).

6.2.3 Patient FM has defective Mac-1 function on neutrophils

Although Patient FM had normal cell surface expression of the β_2 integrins, it was possible that the LAD-1 symptoms were due to the inability of these integrins to bind ligand. Function of the β_2 integrin Mac-1 was assessed by neutrophil binding to plates coated with the ligand fibrinogen. A variety of stimuli were used and all induced neutrophils from a control donor to bind to fibrinogen in a β_2 integrin-dependent manner (Fig. 6.3). The only stimulus to induce Patient FM neutrophils to bind, however, was the β_2 activating mAb KIM185. Therefore, the ability of Mac-1 on Patient FM's neutrophils to bind to fibrinogen was impaired.

6.2.4 Patient FM has defective LFA-1 function on T cells

The function of LFA-1, another β_2 integrin, was tested by T cell adhesion to plates coated with the LFA-1 ligands ICAM-1 and ICAM-3. All the stimuli tested induced LFA-1-mediated T cell binding to both ICAM-1 and ICAM-3 when cells from a control donor were used (Fig. 6.4 A and B). When T cells from Patient FM were tested, however, only Mg^{2+} /EGTA induced adhesion to ICAM-1 (Fig. 6.4 A),

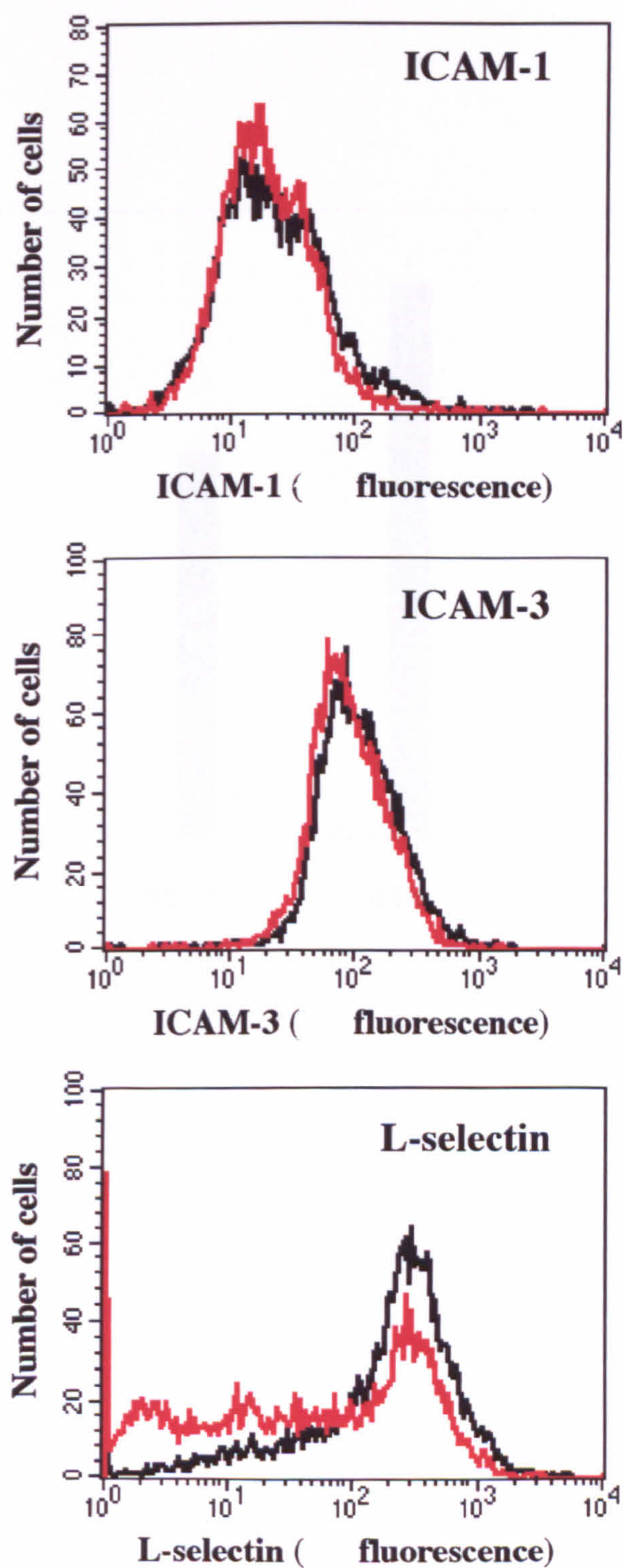


Figure 6.2 Expression of other cell surface molecules on T cells

Expression of ICAM-1 (15.2), ICAM-3 (CH3.1) and L-selectin (LAM1.3) on control (—) and Patient FM (—) T cells . Data are from one experiment (n=2).

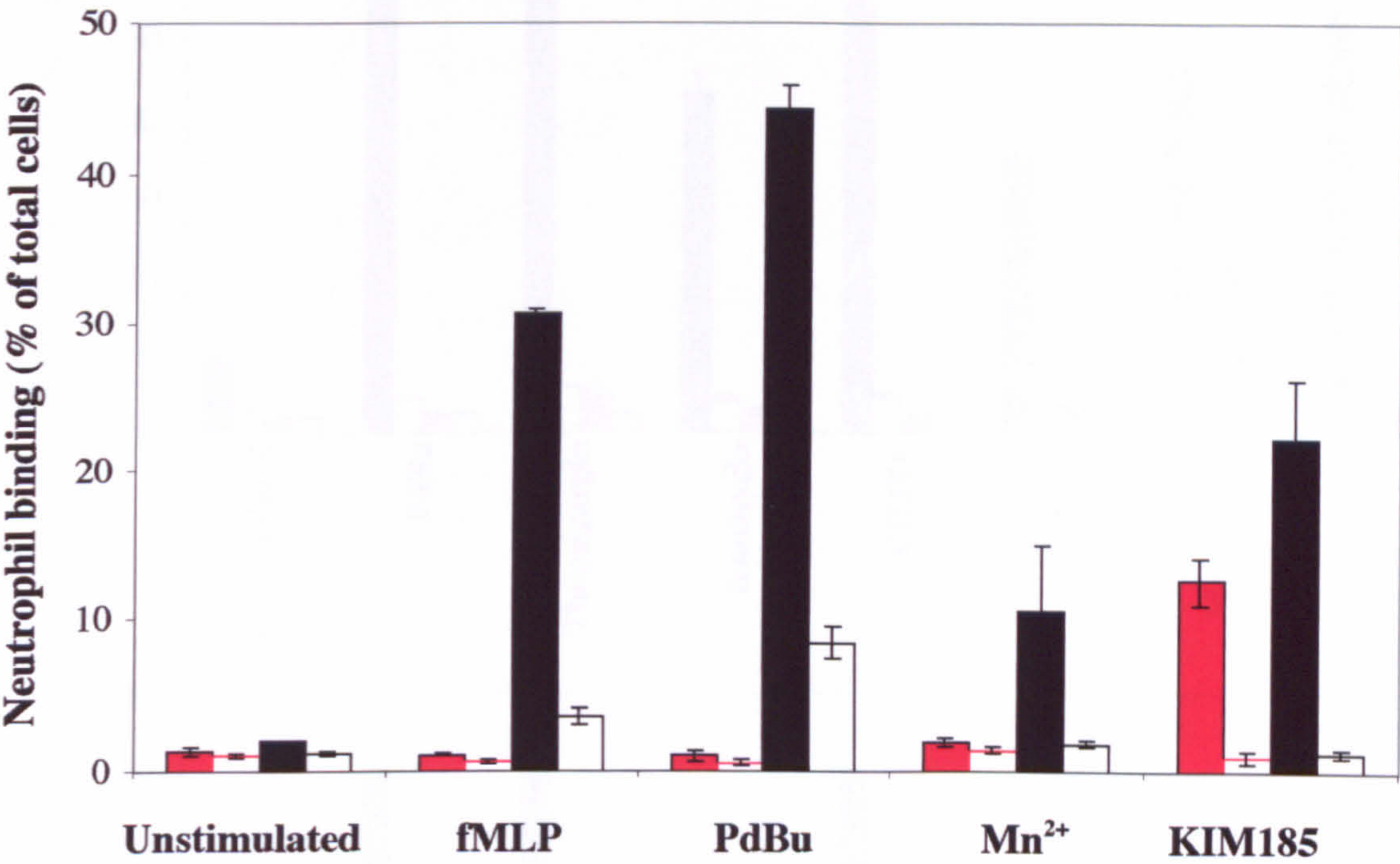


Figure 6.3 Neutrophil binding to fibrinogen

The ability of neutrophils from Patient FM (■) and from a control donor (■) to bind to fibrinogen coated plates when stimulated with 100 nM fMLP, 50 nM PdBu, 0.5 mM MnCl₂, or 5 mM MgCl₂/1 mM EGTA for 30 min RT. The presence of CD18 mAb IB4 at 10 µg/ml inhibits adhesion of Patient FM (□) and control (□) cells. Data (mean of triplicates ± S.D.) from one representative experiment (n=2) are shown.

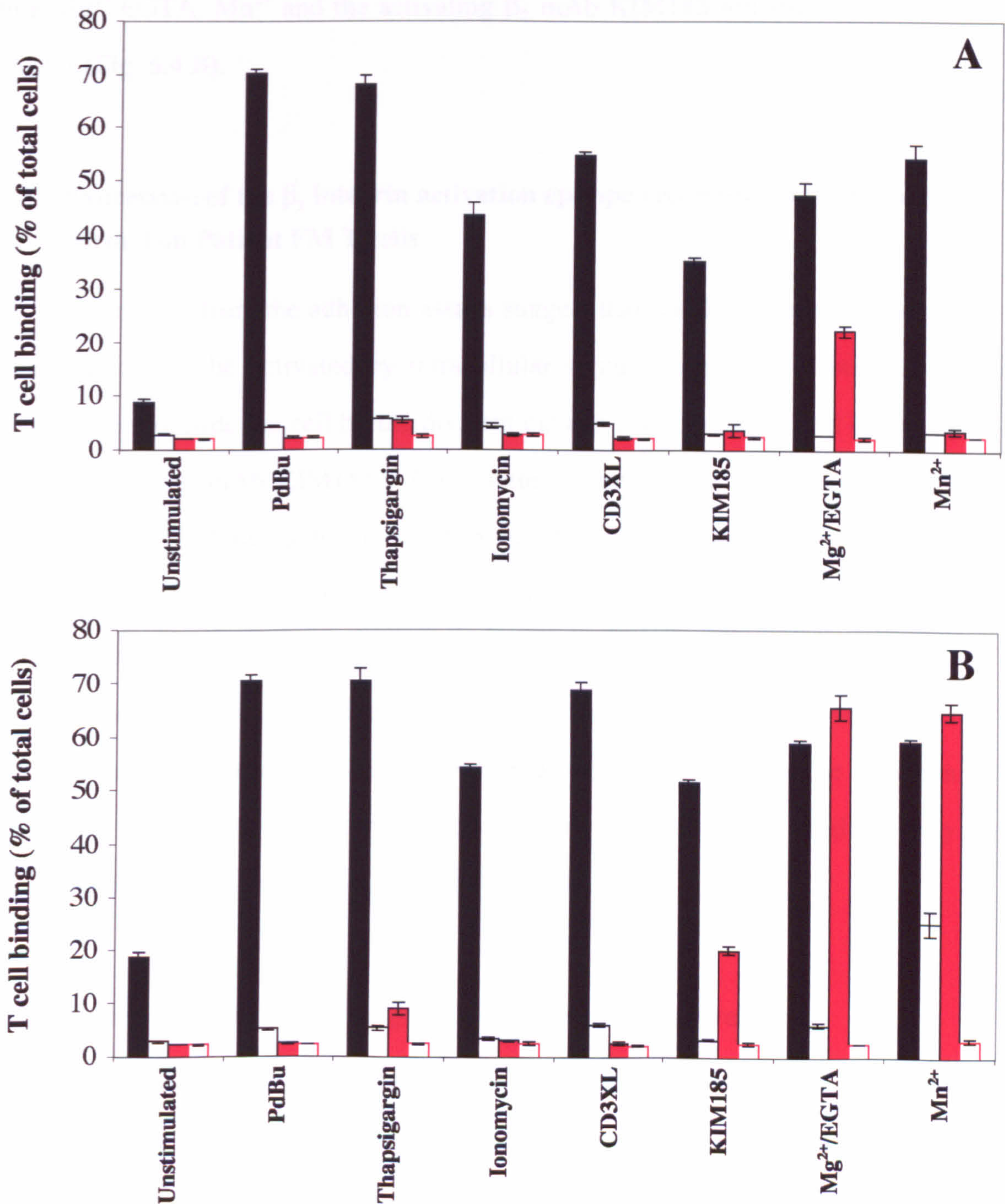


Figure 6.4 T cell binding to ICAM-1 and ICAM-3
The ability of T cells from Patient FM (■) and from a control donor (■) to bind to **A: ICAM-1** and **B: ICAM-3** when stimulated with 50 nM PdBu, 5 μ M thapsigargin, 1 μ M ionomycin, 10 μ g/ml UCHT-1, 10 μ g/ml KIM185, 5 mM Mg²⁺/1 mM EGTA or 0.5 mM Mn²⁺ for 30 min at 37°C. The presence of anti-LFA-1 mAb 38 at 10 μ g/ml inhibits adhesion of Patient FM (□) and control (□) cells. Data (mean of triplicates \pm S.D.) from one representative experiment (n=3) are shown.

while Mg^{2+} /EGTA, Mn^{2+} and the activating β_2 mAb KIM185 induced adhesion to ICAM-3 (Fig. 6.4 B).

6.2.5 Expression of the β_2 integrin activation epitope recognised by mAb 24 is normal on Patient FM T cells

The results from the adhesion assays suggest that the β_2 integrins on Patient FM's cells cannot be activated by intracellular signalling pathways, but can be activated from outside the cell by the divalent cations Mg^{2+} and Mn^{2+} and by the β_2 integrin activating mAb KIM185. These latter forms of stimulation all activate LFA-1 by directly binding to the integrin ectodomain and inducing a structural change to a higher affinity conformation that is recognised by mAb 24. The expression of the mAb 24 epitope on T cells was investigated by flow cytometry. The presence of Mn^{2+} at 37°C induced equivalent levels of 24 binding on T cells from a control donor and from Patient FM (Fig. 6.5). Therefore, as the adhesion assays suggest, stimulation from outside the cell can induce the high affinity conformation of the β_2 integrins on Patient FM's cells.

6.2.6 Patient FM has defective $\alpha_4\beta_1$ and $\alpha_5\beta_1$ function on T cells

As a control, the function of the β_1 integrins on T cells was examined. It was a surprise to find that, whereas all the stimuli tested induced adhesion of control T cells, Patient FM's T cells only adhered to VCAM-1 when stimulated by Mg^{2+} , Mn^{2+} or the β_1 activating mAb TS2/16 (Fig. 6.6 A). These activating agents all act on the integrin ectodomain. T cell adhesion to VCAM-1 is mediated by $\alpha_4\beta_1$ and therefore, in a similar manner to the β_2 integrins, intracellular signalling pathways fail to activate $\alpha_4\beta_1$.

To test whether this defect in intracellular signalling also affected $\alpha_5\beta_1$ function, T cell binding to fibronectin, which is mediated by a combination of $\alpha_4\beta_1$

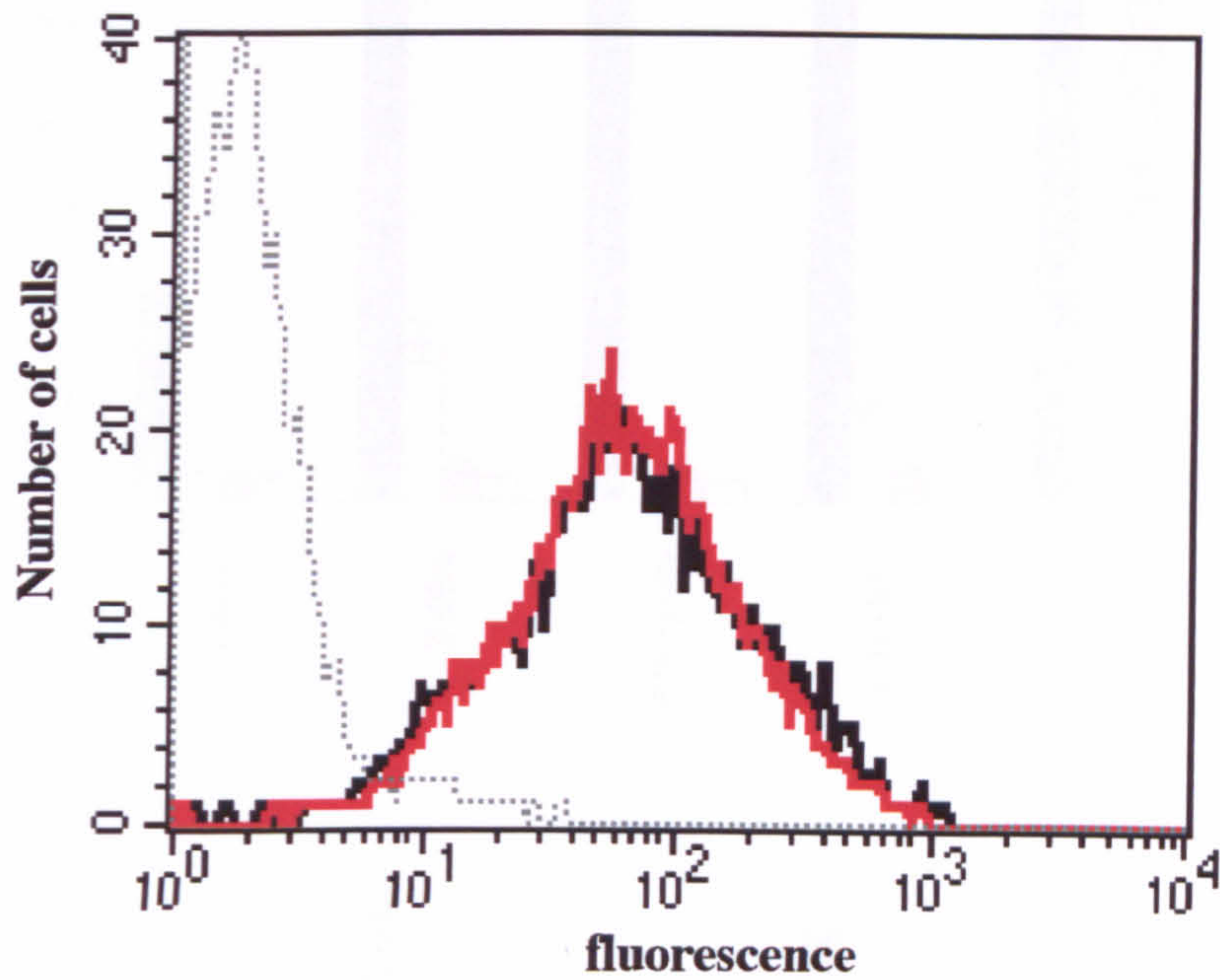


Figure 6.5 Expression of the mAb 24 epitope on T cells

Control (—) and Patient FM (—) T cells incubated with mAb 24 at 10 $\mu\text{g/ml}$ for 30 min at 37°C in the presence of 500 μM MnCl_2 . Control T cells incubated with mAb 24 at 4°C in the presence of 1 mM EGTA(—) are shown as a negative control. Data are from a representative experiment (n=3).

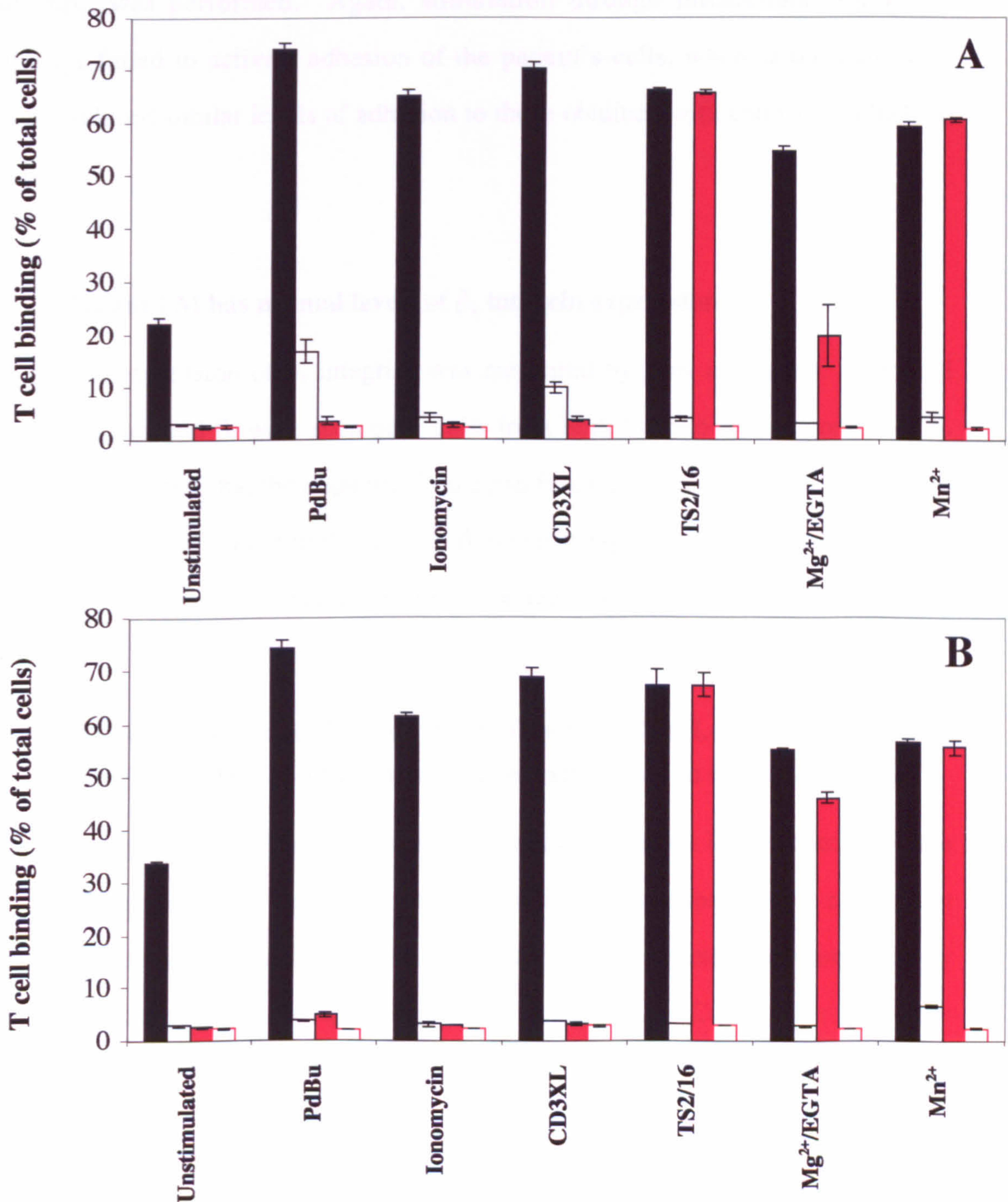


Figure 6.6 T cell binding to VCAM-1 and fibronectin

The ability of T cells from Patient FM (■) and from a control donor (■) to bind to **A:** VCAM-1 and **B:** fibronectin when stimulated with 50 nM PdBu, 5 μ M thapsigargin, 1 μ M ionomycin, 10 μ g/ml UCHT-1, 10 μ g/ml TS2/16, 5 mM Mg²⁺/1 mM EGTA or 0.5 mM Mn²⁺ for 30 min at 37°C. Binding of Patient FM (□) and control (□) cells is inhibited by anti- α_4 mAb HP2/1 at 10 μ g/ml for VCAM-1 and a combination of anti- α_4 mAb HP1/2 and anti- α_5 mAb SAM-1 for fibronectin. Data (mean of triplicates \pm S.D.) from one representative experiment (n=3) are shown.

and $\alpha_5\beta_1$, was performed. Again, stimulation through intracellular signalling pathways failed to activate adhesion of the patient's cells, whereas the outside-in stimuli induced similar levels of adhesion to those obtained with control T cells (Fig 6.6 B).

6.2.7 Patient FM has normal levels of β_1 integrin expression

The expression of β_1 integrins was measured by flow cytometry. Identical levels of α_4 , α_5 and β_1 were seen on T cells from both the patient and control (Fig. 6.7). This confirms that the impaired β_1 integrin function seen in the adhesion assays was not due to a difference in the level of β_1 integrin expression, but is more likely to be due to a defect in the signalling pathway that leads to integrin activation.

6.2.8 Patient FM's neutrophils are able to flux Ca^{2+} , shed L-selectin, upregulate Mac-1 and produce a superoxide burst in response to fMLP

To verify that the lack of integrin activation on Patient FM's leukocytes was not due to a gross signalling defect, some preliminary experiments were carried out to test neutrophil responses to fMLP. One of the early events common to many signalling pathways is the mobilisation of intracellular Ca^{2+} . The patient's neutrophils were able to elevate $[\text{Ca}^{2+}]_i$ in response to fMLP (Fig. 6.8 A), and in response to the Ca^{2+} mobilising agent thapsigargin (Fig. 6.8 B).

Other responses that are characteristic of neutrophil activation by fMLP were also tested. L-selectin shedding (Fig. 6.9 A), the upregulation of Mac-1 (Fig. 6.9 B) and superoxide burst (Fig. 6.9 C) were found to occur at a similar extent in neutrophils from Patient FM and from a control. Therefore, despite the fact that fMLP cannot stimulate Patient FM's neutrophils to bind to fibrinogen (Fig. 6.3), these preliminary experiments (n=1) indicate that fMLP does activate the patient's neutrophils.

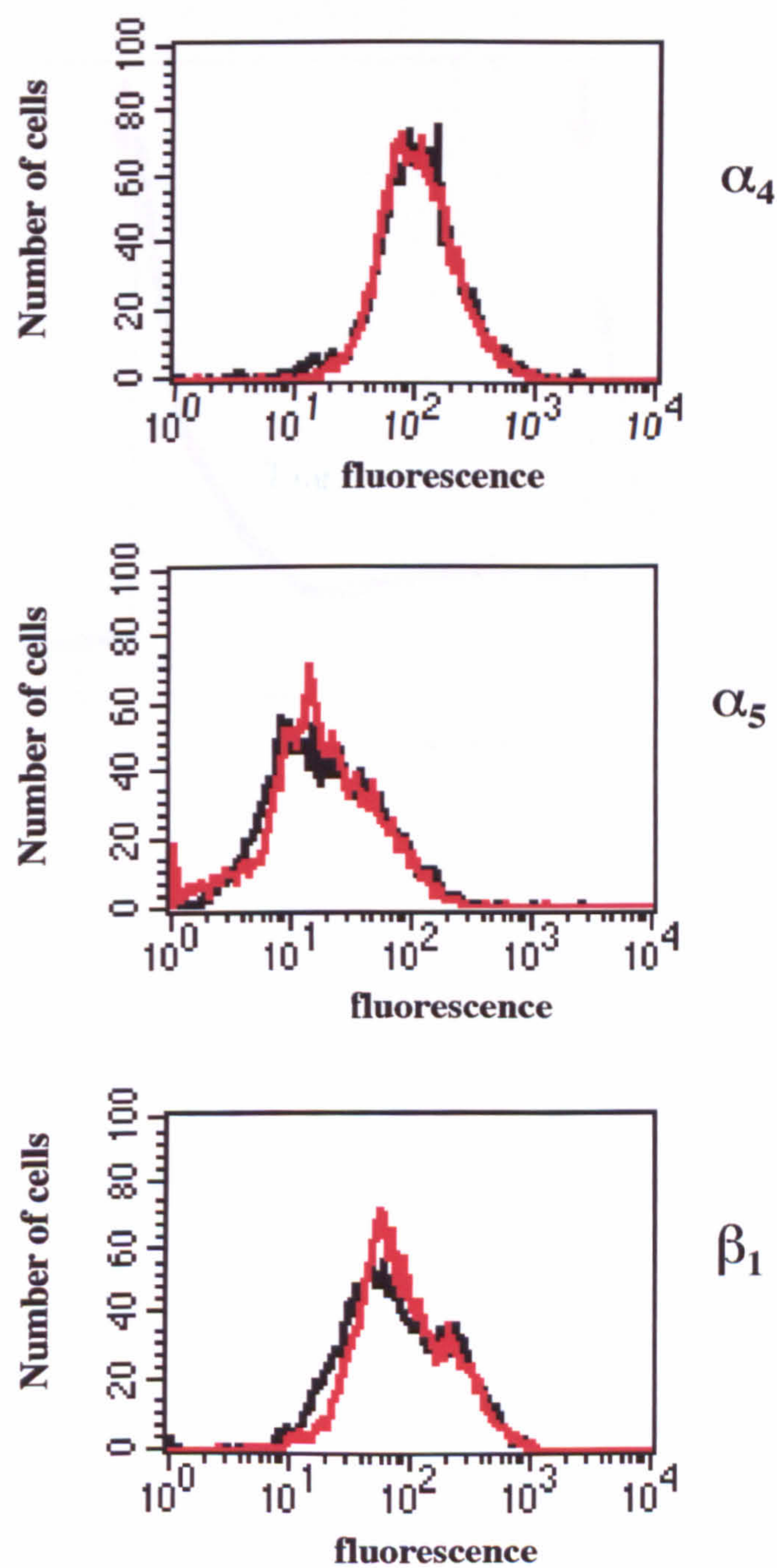


Figure 6.7 Expression of the β_1 integrins on T cells

Expression of α_4 (HP1/2), α_5 (SAM-1) and β_1 (P5D2) on control (—) and Patient FM (—) T cells. Data are from one experiment (n=2).

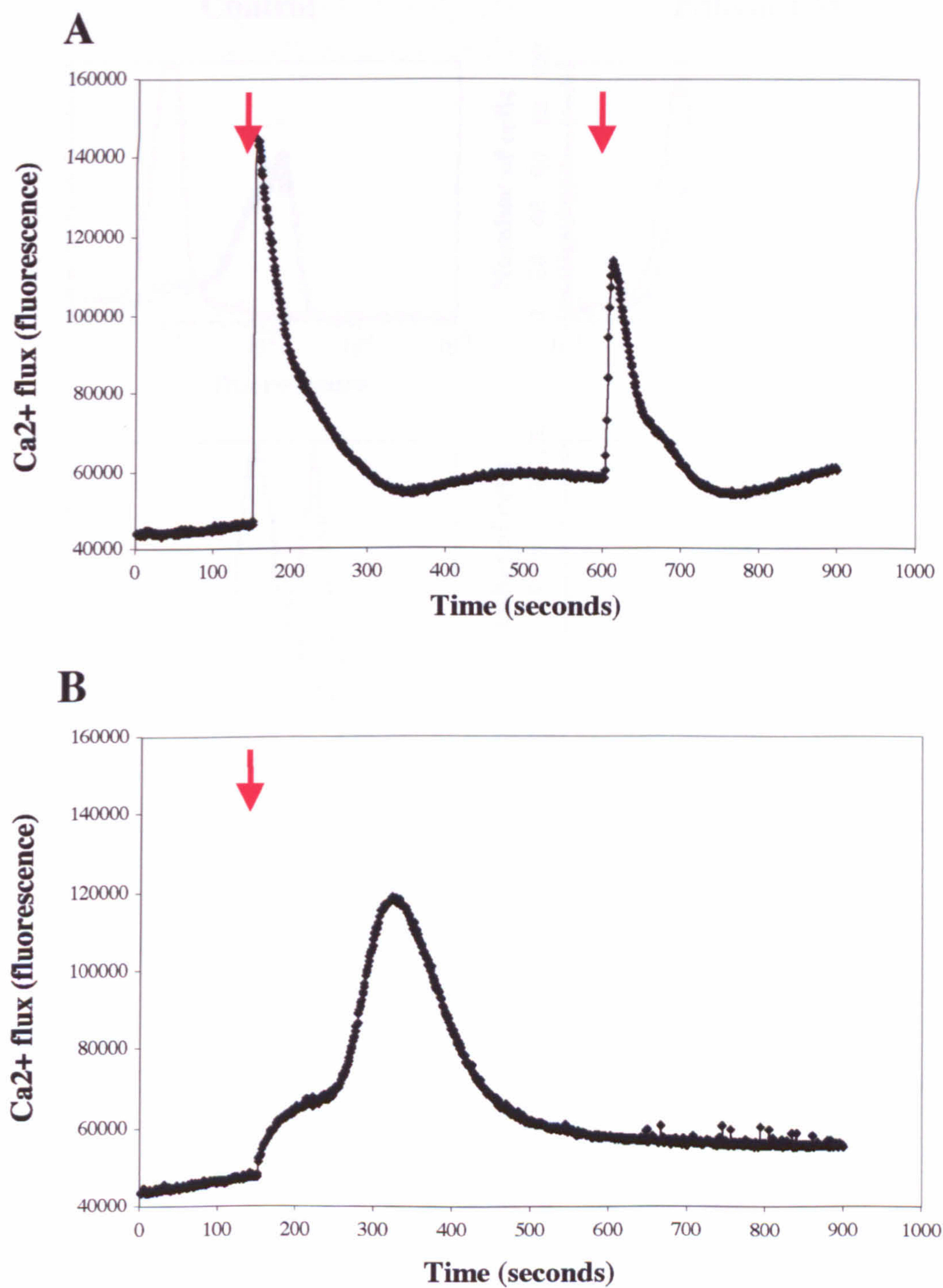


Figure 6.8 Intracellular Ca^{2+} mobilisation in response to fMLP and thapsigargin stimulation of neutrophils from Patient FM
Intracellular Ca^{2+} flux in Fluo-3 labelled neutrophils from Patient FM stimulated with **A:** 0.1 μM fMLP and **B:** 5 μM thapsigargin at the times indicated by the arrows. Increases in $[\text{Ca}^{2+}]_i$ were detected by an increase in fluorescence of Fluo-3 and are represented by arbitrary units. This is a preliminary experiment (n=1).

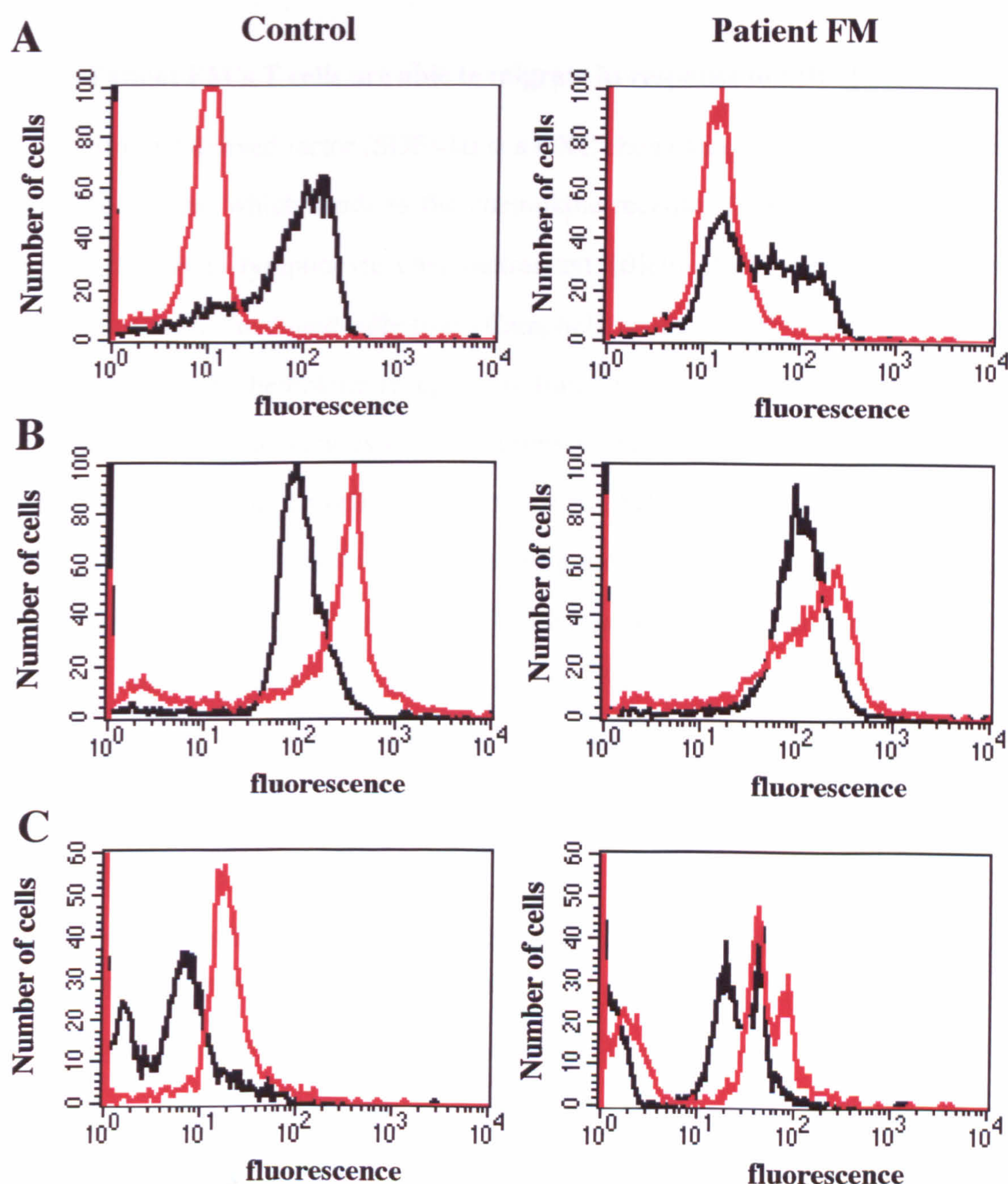


Figure 6.9 The effect of fMLP on L-selectin shedding, Mac-1 upregulation and superoxide burst in neutrophils

Control and Patient FM neutrophils were incubated for 30 min at 37°C without stimulation (—) or with 0.1 μ M fMLP (—) and tested for **A:** expression of L-selectin (mAb LAM1.3), **B:** expression of Mac-1 (mAb ICRF44) and **C:** superoxide burst. Increases in superoxide were detected by an increase in fluorescence of dihydrorhodamine. This is a preliminary experiment (n=1).

6.2.9 Patient FM's T cells are able to migrate in response to SDF-1 α

Stromal derived factor (SDF)-1 α is a CXC chemokine produced in secondary lymphoid organs, which binds to the chemokine receptor CXCR4. SDF-1 α is a highly efficacious lymphocyte chemoattractant (Bleul et al., 1996), capable of inducing integrin mediated adhesion (Campbell et al., 1998). To test whether signalling through chemokine receptors is functioning in Patient FM's cells, the response of T cells to various concentrations of SDF-1 α was measured using a Transwell system, with the cells in the top well and SDF-1 α in the bottom well (Fig. 6.10). Patient FM's T cells were able to respond to SDF-1 α . However, whereas approximately 70% of control input cells had migrated within 90 min toward 10 nM SDF-1 α , only approximately 35% of Patient FM's cells had migrated. The spontaneous migration without stimulus was consistently lower for Patient FM's cells than for the control's.

6.2.10 Patient FM has normal expression of a number of cytoskeletal proteins reported to associate with integrins

The link between integrins and the cytoskeleton is critical for their function and it is therefore possible that the dysfunction of integrins on Patient FM's leukocytes is due to a defect in a cytoskeletal protein. A number of cytoskeletal proteins have been reported to associate with integrins. Many of these interactions are probably not direct, are likely to be integrin and cell type specific, and may also depend on the activation state of both the cell and integrin. The cytoskeletal associations of integrins on leukocytes are therefore ill-defined. As an initial attempt to address whether any of these associations are defective in Patient FM's T cells, lysates were blotted for some of the most commonly reported integrin-associated

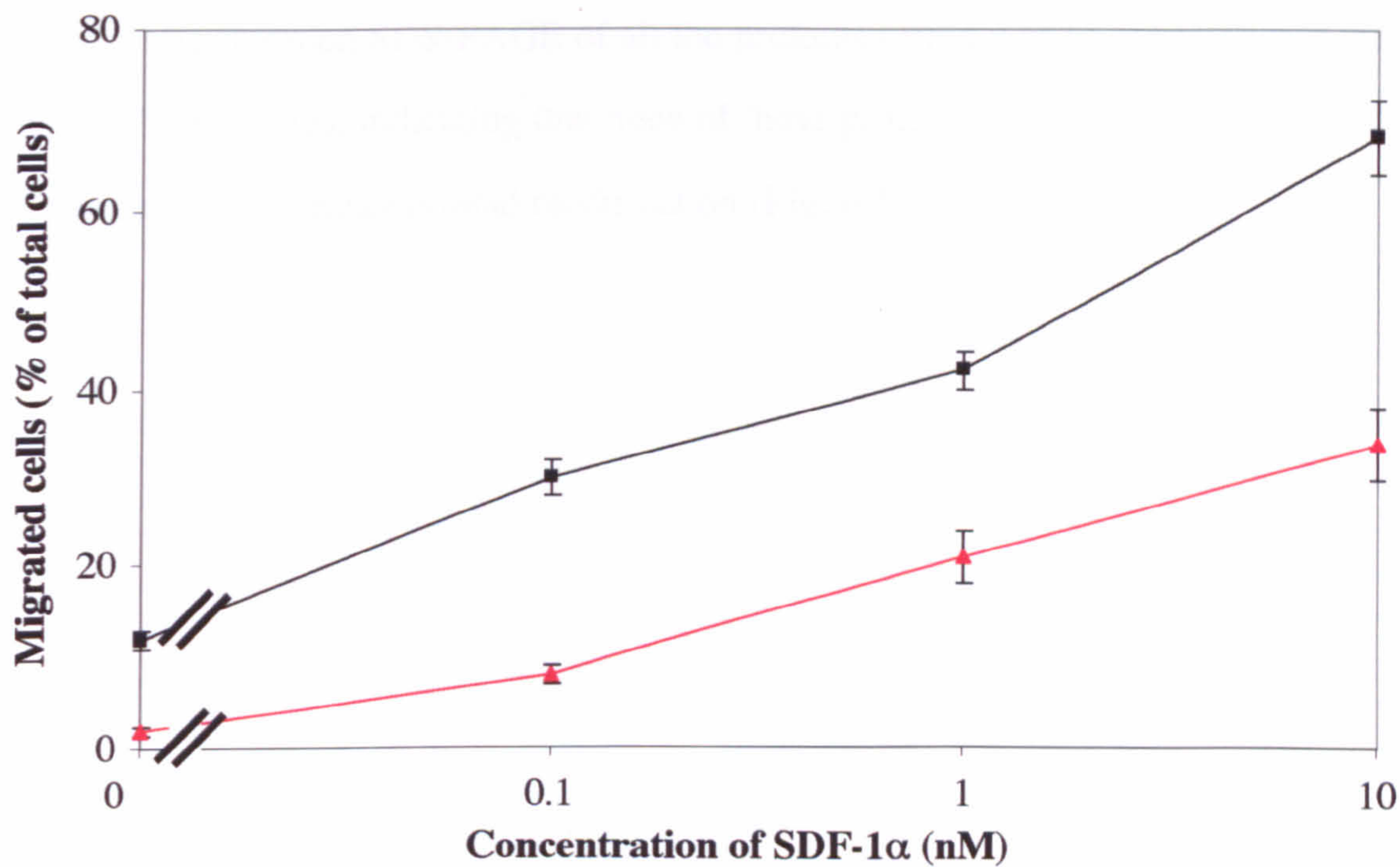


Figure 6.10 T cell migration in response to SDF-1α
T cells from Patient FM (▲) and from a control donor (■) were placed in the upper well of a Transwell plate and allowed to migrate for 90 min at 37°C in response to 0-10 nM SDF-1α in the lower chamber. Data (mean of triplicates ± S.D.) from one representative experiment (n=3) are shown.

cytoskeletal proteins. As can be seen in **Figure 6.11**, filamin, talin, α -actinin, vinculin, ezrin and actin are all present in the patient's lysate and are expressed at equivalent levels in the control lysate. This was also the case for the LFA-1 alpha subunit. Migration on SDS-PAGE of all the proteins tested was as expected and the same for both lysates, indicating that none of these proteins had been cleaved or had differences in post-transcriptional modification (**Fig. 6.11**).

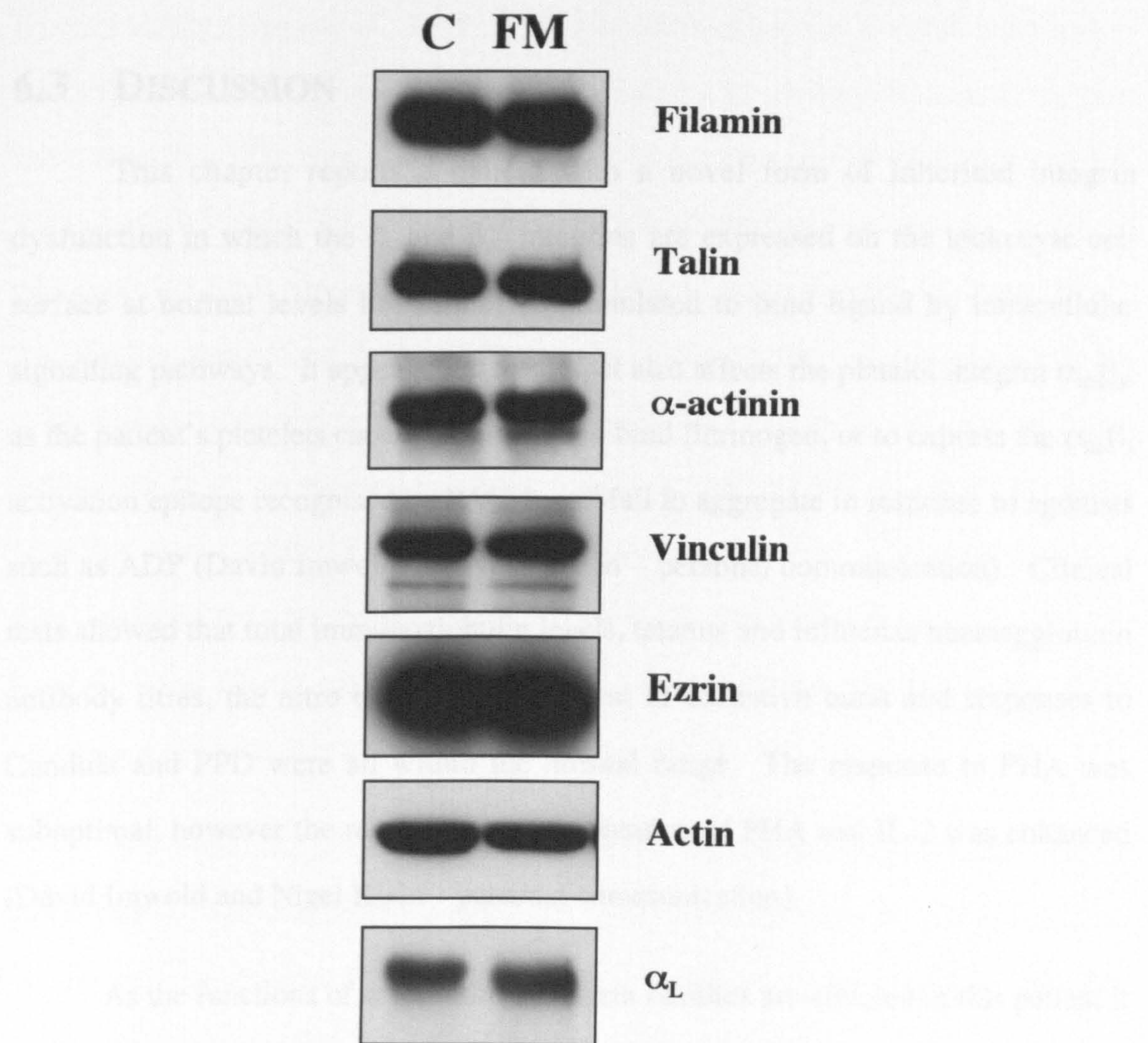


Figure 6.11 Western blots of T cell lysates from a control donor and Patient FM immunostained for cytoskeletal proteins

T cell lysates from a control donor (C) and Patient FM were blotted for the cytoskeletal proteins indicated and also for the LFA-1 α subunit (n=2).

6.3 DISCUSSION

This chapter reports a patient with a novel form of inherited integrin dysfunction in which the β_1 and β_2 integrins are expressed on the leukocyte cell surface at normal levels but cannot be stimulated to bind ligand by intracellular signalling pathways. It appears that the defect also affects the platelet integrin $\alpha_{\text{IIb}}\beta_3$, as the patient's platelets cannot be induced to bind fibrinogen, or to express the $\alpha_{\text{IIb}}\beta_3$ activation epitope recognised by PAC-1, and fail to aggregate in response to agonists such as ADP (David Imwold and Nigel Klein – personal communication). Clinical tests showed that total immunoglobulin levels, tetanus and influenza haemagglutinin antibody titres, the nitro blue tetrazolium test of oxidative burst and responses to *Candida* and PPD were all within the normal range. The response to PHA was suboptimal, however the response to a combination of PHA and IL-2 was enhanced (David Imwold and Nigel Klein – personal communication).

As the functions of at least three integrin families are affected in this patient it is unlikely that the dysfunction is due to mutations in the integrin α or β subunits themselves. It is more probable that the faulty gene encodes a protein that is critical for integrin function and that expression of this protein is restricted to haematopoietic cells. This latter conjecture is based on evidence from knockout mice which shows that integrins are essential for embryo survival: for instance both the α_4 and β_1 integrin knockouts are embryonic lethal (Fassler and Meyer, 1995; Yang et al., 1995). It would therefore be expected that the patient would not have survived if the functions of β_1 integrins on all cells were affected.

Three other patients with non-classical forms of LAD-1 have been described. The first expressed ~60% of the normal levels of β_2 integrins, but despite this had no β_2 integrin function. This patient was a compound heterozygote with a mutation in one allele that prevented expression and a mutation in the other allele that prevented

ligand binding (Hogg et al., 1999). The second case had the clinical indicators of LAD-1 and the β_2 integrins were non-functional, but no abnormalities in the β_2 gene were detected. With time, defects in β_3 integrin function also became apparent, but, in contrast to Patient FM, β_1 integrins were not affected (Kuijpers et al., 1997). It was speculated that the problem lay in an integrin-associated protein rather than in the integrins themselves. In the third case, the clinical features of LAD-1 were found to be due to a mutation in the gene encoding the haematopoietic cell-specific small GTPase Rac2, which resulted in it being unable to bind GTP (Ambruso et al., 2000; Williams et al., 2000). Patient FM has elevated numbers of lymphocytes and an intact superoxide burst, distinguishing her from the Rac2 defective patient. For the Rac2 defective patient, and also for the Rac2 knockout mouse (Roberts et al., 1999), the dysfunctions described are restricted to neutrophils.

The signalling and cytoskeletal changes leading to integrin activation on leukocytes are not well defined. Although intracellular signalling could not activate the integrins on Patient FM's leukocytes and platelets, other functions were still intact. For example, fMLP did not stimulate neutrophil binding to fibrinogen but in preliminary experiments did induce upregulation of Mac-1 expression, L-selectin shedding and superoxide burst. This suggests that the defect is in a protein that is proximal to the integrin. A number of cytoskeletal proteins that have been reported to be associated with integrins (see **Chapter Five**) were present at normal levels in Patient FM T cells. This result indicates that the dysfunction is not due to lack of expression of one of these proteins, but does not preclude the possibility that a mutation in one of these proteins is disrupting its function. One obvious candidate for the abnormal protein is the guanine nucleotide exchange factor cytohesin-1. Cytohesin-1 is prevalently expressed in immune cells and interacts with the β_2 subunit cytoplasmic tail to control LFA-1 function in Jurkat cells (Kolanus et al., 1996). However, cytohesin-1 does not interact with the β_1 subunit and has no effect on β_1 integrin function in the Jurkat T cell line (Kolanus et al., 1996), which

therefore diminishes the likelihood of it being implicated. The small GTPase Rap-1 has also been demonstrated to lie in the signalling pathway leading to LFA-1, and also Mac-1, activation (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000), however Rap-1 is ubiquitously expressed.

The β_1 and β_2 integrins play an important role in the extravasation of leukocytes to sites of injury or infection and in the homing of lymphocytes to tissues, particularly the secondary lymphoid organs. Leukocyte recruitment from the circulation to tissue, across vascular endothelium (or high endothelial venules of secondary lymphoid organs) involves a cascade of events (see **Figure 1.9**). First, leukocytes roll along the endothelium mediated by L-selectin on the leukocyte and P- and E-selectin on the endothelium. Next, chemokines bound to the endothelium activate their receptors on leukocytes resulting in L-selectin shedding, integrin activation, and thus integrin-mediated firm adhesion of the leukocytes to the endothelium. Finally, integrins mediate diapedesis of the leukocytes between the endothelial cells to the extravascular space (reviewed in (Carlos and Harlan, 1994; Springer, 1994)). Typically, LAD-1 patients have elevated numbers of circulating neutrophils, as these cells fail to adhere or to migrate across the endothelium. This results in the failure of LAD-1 patients to clear bacterial infections. With the additional defect of lack of β_1 function, it would be expected that Patient FM would have further problems. In experiments with knockout mice, lack of β_2 integrins can be somewhat compensated for by the β_1 integrins (Berlin-Rufenach et al., 1999). Without both families of integrins it would be expected that lymphocyte trafficking would be severely impaired. This may partially explain why Patient FM has a vastly elevated number of circulating lymphocytes, a feature that is not so prominent in classic LAD-1 patients. Additionally, lack of integrin function may also affect haematopoiesis. The regulation of haematopoiesis is not well understood but requires both soluble factors and interaction of the stem cells/progenitors with the stromal cells in the bone marrow and thymus. The integrins are one of the families

of adhesion molecules predicted to be involved in the homing of progenitors to the bone marrow and the retention of progenitors in the bone marrow. Indeed, in mice with conditional α_4 null B cells, these cells exit the bone marrow prematurely, before full differentiation and proliferation has taken place (Arroyo et al., 1996). Signalling through the integrins may also affect cell proliferation and cell survival. However whether increased generation, increased lifespan, increased proliferation or decreased migration of mature cells creates the elevated lymphocyte numbers remains to be investigated.

The potential to correct inherited genetic disorders of blood cells by introducing a normal copy of the faulty gene into haematopoietic stem cells has been discussed for some years. Unfortunately human stem cell gene therapy trials have proved disappointing as the efficiency of gene transfer has been low and of short duration. Development of techniques for stem cell gene transfer involving the use of a fragment of fibronectin to aid co-localisation of the retroviral vector and the target stem cell are improving transduction levels (Abonour et al., 2000). A recent report of the successful use of stem cell gene therapy to treat two children with Severe Combined Immunodeficiency (SCID) used this protocol (Cavazzana-Calvo et al., 2000). Although it is too early to determine whether this therapy will give long term alleviation from SCID, the results are encouraging and the patients have been able to leave sterile isolation. A potential problem of using gene therapy to correct integrin dysfunction is that integrins do not function normally when transfected into cells. It has been reported, however, that retroviral mediated transfer of the CD18 gene allowed LAD-1 stem cells to gain CD18 dependent functions *ex vivo* (Bauer et al., 1998). Recently, the integrin β_3 gene under the control of the α_{mb} promoter was introduced into CD34⁺ peripheral blood stem cells from patients with Glanzmann's Thrombasthenia in order to drive megakaryocyte-targeted expression of the normal β_3 subunit. The transduced megakaryocytes were able to retract a fibrin clot, demonstrating $\alpha_{mb}\beta_3$ function (Wilcox et al., 2000). These promising developments

in gene therapy intensify the importance of identifying the molecular basis behind inherited diseases. Currently, bone marrow transplantation is the only curative treatment for LAD-1.

In summary, a defect in the signalling pathway which leads to integrin activation on leukocytes and platelets, rather than a defect in the integrins themselves, appears to be responsible for the LAD-1- and Glanzmann's Thrombasthenia-like symptoms of Patient FM. Patient FM therefore has a non-classical form of integrin dysfunction. The affected pathway is probably restricted to cells of haematopoietic origin and it is likely that events proximal to the integrin are affected. The defects described here demonstrate that detection of integrins on the cell surface does not necessarily exclude a diagnosis of LAD-1 or Glanzmann's Thrombasthenia.

CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

7.1 SUMMARY

Integrins facilitate many leukocyte interactions in the immune system. For example, they are involved in leukocyte migration to peripheral lymph nodes and inflammatory sites, antigen presentation and cytotoxic killing. To prevent inappropriate induction of these processes, it is crucial that the interaction of the integrins with their ligands is tightly controlled. Under normal conditions, the integrins on leukocytes are inactive, but engagement of the T and B cell receptors, exposure to cytokines and chemokines, and cross talk from other cell surface adhesion molecules, all result in rapid integrin activation. However, little has been known about the detailed molecular mechanisms that regulate integrin activation on leukocytes. The findings of this thesis contribute to the understanding of the processes of integrin activation and ligand interaction. Specifically, the activation of the leukocyte integrin LFA-1 on T cells and its interaction with its ligands ICAM-1 and ICAM-3 were investigated.

7.1.1 LFA-1 binding to the ICAMs

ICAM-1, ICAM-2 and ICAM-3 are closely related members of the immunoglobulin superfamily and all are ligands for LFA-1. Recently, the structures of domains 1 and 2 of ICAM-1 and ICAM-2 have been solved (Casasnovas et al., 1997; Casasnovas et al., 1998b). These domains are Ig-like, each composed of two β sheets, one containing the A', C, F and G strands, the other the A, B, D and E strands. The C and D strands form an edge to the domain. Residues Glu³⁴, Met⁶⁴, Tyr⁶⁶ and Gln⁷³ of ICAM-1 are essential for interaction with LFA-1 (Staunton et al.,

1990). Glu³⁴ is the last residue in the C strand in domain 1 of ICAM-1, Met⁶⁴ and Tyr⁶⁶ are in strand F and Gln⁷³ lies in strand G. Thus, the CFG face of domain 1 of ICAM-1 is a binding site for LFA-1. In **Chapter 3**, the binding site for LFA-1 on ICAM-3 was characterised. The results demonstrate that Glu³⁷, Leu⁶⁶, Ser⁶⁸ and Gln⁷⁵ in domain 1 of ICAM-3 are critical for this interaction. The structure of ICAM-3 has not yet been determined, but is predicted to be similar to that of ICAM-1. Thus, the results in **Chapter 3** indicate that LFA-1 makes contact with residues on the CFG face of ICAM-3, in a similar position to those on ICAM-1. Extensive mutagenesis has recently defined the LFA-1 binding site in ICAM-2 (Casasnovas et al., 1999). It is oblong in shape and extends diagonally across the CFG face and CD edge. The pattern that has emerged from all these results is that LFA-1 has a similar binding footprints on all three ICAMs.

It is predicted that Glu³⁴ of ICAM-1, or Glu³⁷ in ICAM-2 and ICAM-3, is central to the interaction with LFA-1. Glu³⁴ of ICAM-1 is essential for binding to the LFA-1 I domain (Stanley and Hogg, 1998), and it has been proposed that it coordinates the metal ion bound to the MIDAS motif (Lee et al., 1995b). The MIDAS face of the I domain would thus make contact with the CFG face of domain 1 of ICAM-1. From the results in **Chapter 3**, it is predicted that ICAM-3 would bind to the LFA-1 I domain in a similar manner to ICAM-1, with Glu³⁷ coordinating the metal ion. In **Chapter 4**, a peptide from the I domain inhibited T cell binding to ICAM-3 without affecting binding to ICAM-1. This indicates that there may be some selectivity to the interaction of the I domain with different ligands. We await co-crystals of the LFA-1 I domain with its various ICAM ligands to confirm these predictions.

It has been questioned whether LFA-1 binding sites exist in ICAM-1 other than the CFG face of domain 1. In **Chapter 3**, mutagenesis of H¹⁵²HG in the EF loop in domain 2 of ICAM-1 disrupted binding to LFA-1. It has subsequently been demonstrated that this mutation prevents binding of the isolated LFA-1 I domain, but

that the I domain does not bind directly to domain 2 (Stanley et al., 2000). We have therefore concluded that ICAM-1 domain 2 plays an important role in binding to LFA-1 but that it is not a direct binding site.

In addition to binding the leukocyte integrins, ICAM-1 serves as a receptor for erythrocytes infected with the malarial parasite, *P. falciparum*. In Kilifi, Kenya a K²⁹/M polymorphism in ICAM-1 was found to be associated with increased susceptibility to cerebral malaria. This was an unexpected finding. How could a polymorphism with such an undesirable consequence have arisen? In **Chapter 3**, ICAM-1 with the K²⁹/M mutation (ICAM-1^{Kilifi}) was shown to support LFA-1-mediated T cell adhesion, but with a lower affinity than wt ICAM-1. It would be interesting to test whether the K²⁹/M mutation has a more severe effect on T cell binding under flow conditions, more accurately mimicking the *in vivo* situation. Our collaborator, D. Altieri, found that binding of ICAM-1^{Kilifi} to fibrinogen was severely impaired compared to wt ICAM-1 binding. By binding to ICAM-1 on the endothelium and Mac-1 on the leukocyte, fibrinogen functions as a bridge for leukocyte-endothelial interactions. Thus, by reducing the affinity of LFA-1 binding and diminishing fibrinogen binding, the ICAM-1^{Kilifi} polymorphism could limit leukocyte transmigration. This could, perhaps, be advantageous when subjected to continuous microbial infection.

7.1.2 LFA-1 affinity regulation

Binding of the divalent cation Mg²⁺ to the LFA-1 ectodomain induces a high affinity form of LFA-1, without requirement for intracellular signalling. This form of LFA-1 is characterised by its ability to bind soluble ICAM-1 and by expression of the epitope recognised by mAb 24 (Stewart et al., 1996). In **Chapter 4**, it was shown that formation of this high affinity form of LFA-1 requires interdomain movement involving the I domain. A peptide corresponding to the β_3 to α_5 loop and α_5 helix of the I domain inhibited transition to the high affinity conformation,

suggesting this region of the domain alters its associations within the molecule allowing higher affinity binding to ligand.

The *in vivo* relevance of regulation of integrin affinity has been questioned. Is this merely an experimental manipulation or are there physiological situations in which the affinity of integrins is altered? Until recently, evidence has been lacking that inside-out signalling can switch LFA-1 from a low affinity to a high affinity conformation. However, two publications in the last year have demonstrated that activation of LFA-1 through a Rap-1-dependent pathway results in increased LFA-1 affinity, as detected by soluble ICAM-1 binding and expression of the mAb 24 epitope (Katagiri et al., 2000; Reedquist et al., 2000). There may also be situations, such as wound healing, where alterations in Mg^{2+} and Ca^{2+} concentration occur that could influence LFA-1 affinity (Grzesiak and Pierschbacher, 1995).

7.1.3 LFA-1 avidity

LFA-1 can be activated to bind its ligands by intracellular signalling initiated by engagement of cell surface molecules, e.g. the TCR. Raising $[Ca^{2+}]_i$ by treatment with ionomycin or thapsigargin, or activating PKC with phorbol ester, has a similar effect. How these signals induce LFA-1 adhesion has been unclear. No increase in LFA-1 affinity can be detected and there is no evidence for a conformational change. In Chapter 4, it was shown that this form of adhesion was more sensitive than Mg^{2+} /EGTA-stimulated adhesion to inhibition by mAbs that recognise the first and last helices of the β I-like domain. It is interesting that in the α subunit I domain the first and last helices lie close together at the bottom of the domain and have been implicated in allosteric control of ligand binding (Kallen et al., 1999).

The process by which raising $[Ca^{2+}]_i$ regulates LFA-1 mediated T cell binding, was investigated in Chapter 5. This process requires the influx of extracellular Ca^{2+} , is susceptible to inhibitors of cytoskeletal reorganisation and

involves the Ca^{2+} -dependent protease calpain. Confocal microscopy revealed that upon treatment of T cells with Ca^{2+} mobilising agents, LFA-1 becomes clustered. It is believed that this clustering enhances the avidity of ligand binding.

There is evidence that restraint by the cytoskeleton maintains LFA-1 in an inactive state (Kucik et al., 1996; Tan et al., 2000; van Kooyk et al., 1999). These results were supported by the use of jasplakinolide, an agent which prevents reorganisation of the actin cytoskeleton. Jasplakinolide prevented LFA-1 clustering and adhesion to ICAM-1. As many cytoskeletal proteins are targets of calpain, we formed a working hypothesis in which (1) in resting cells LFA-1 is tethered to the cytoskeleton, (2) raised $[\text{Ca}^{2+}]_i$ activates calpain, (3) this protease then cleaves an LFA-1-associated cytoskeletal protein, (4) the LFA-1 released from restraint by the cytoskeleton is free to move in the membrane and redistribute into clusters, and (5) these clusters strengthen binding to ICAM-1. It therefore seemed pertinent to investigate LFA-1 association with the cytoskeleton. These experiments proved to be technically difficult, however, so conclusive results were not obtained. Attempts were also made to identify the specific target of calpain, but this remains elusive.

7.1.4 A model for LFA-1 activation

The results in Chapters 4 and 5 lead to three possible models for how changes in LFA-1 affinity and avidity could integrate to lead to ligand binding (Fig. 7.1). The first option (Fig. 7.1 A) is that (1) LFA-1 exists in a low affinity, unclustered form on resting cells, (2) intracellular signalling events lead to clustering of LFA-1, (3) interaction with ligand induces a conformational change in LFA-1, which increases the affinity of ligand binding. In the second model (Fig. 7.1 B), (1) LFA-1 exists in a low affinity, unclustered form on resting cells, (2) intracellular signalling events lead to both LFA-1 clustering and a change to the higher affinity LFA-1 conformation, (3) ligand binding occurs through LFA-1 clusters containing a combination of low and high affinity LFA-1. The third possibility (Fig. 7.1 C) is

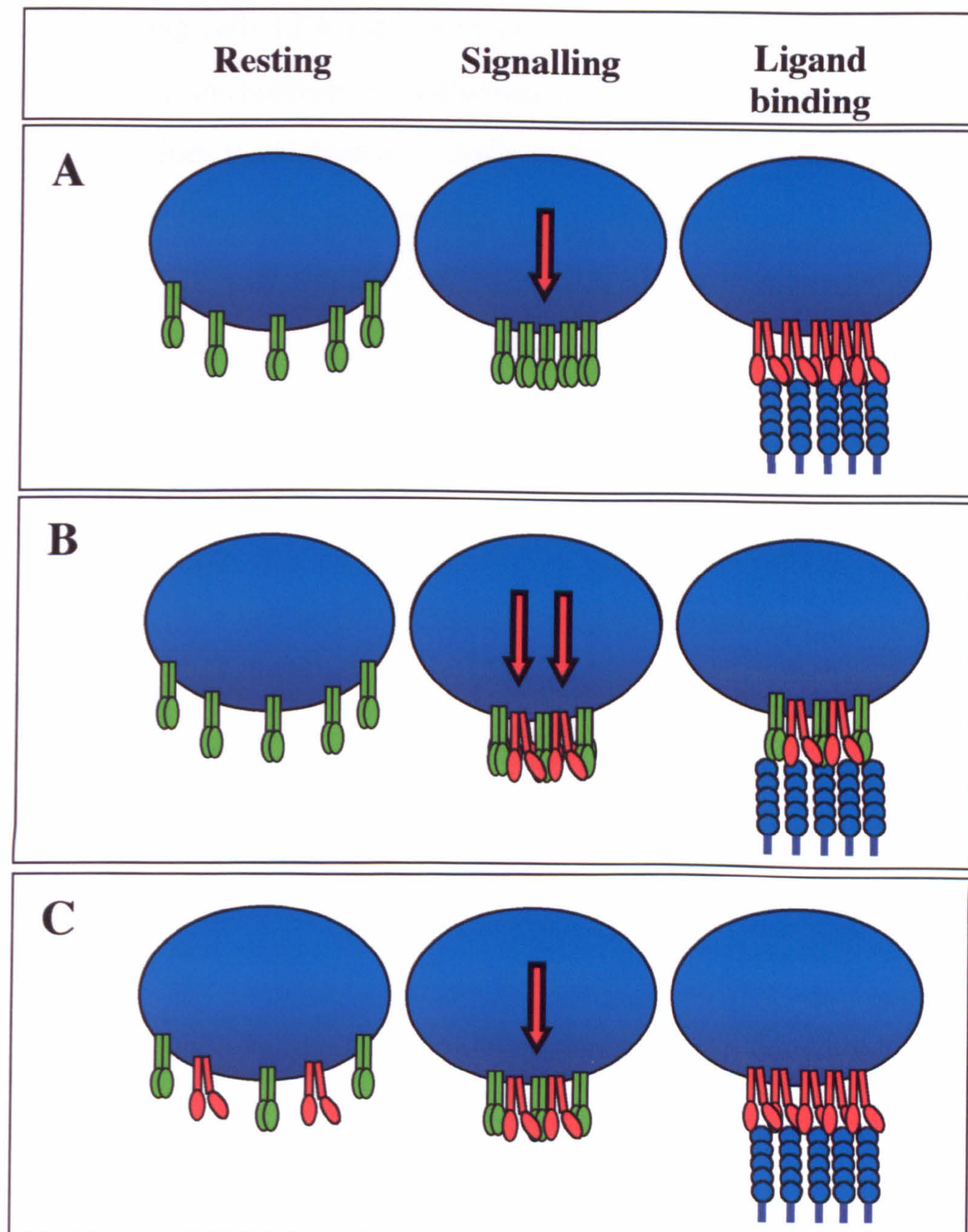


Figure 7.1 Three models for how changes in LFA-1 affinity and avidity could integrate to lead to ligand binding

A: LFA-1 exists in a low affinity conformation (green) and is not clustered on resting cells. Intracellular signalling leads to clustering of LFA-1. Interaction with ligand induces the high affinity conformation of LFA-1 (red). **B** LFA-1 exists in a low affinity conformation (green) and is not clustered on resting cells. Intracellular signalling leads to both LFA-1 clustering and a change to the higher affinity LFA-1 conformation (red). Ligand binding occurs through LFA-1 clusters containing a combination of low and high affinity LFA-1. **C:** On resting cells LFA-1 exists in an equilibrium of low (green) and high affinity (red) conformations. Intracellular signalling leads to LFA-1 clustering, then interaction with ligand stabilises the high affinity conformation of LFA-1.

that (1) on resting cells LFA-1 exists in an equilibrium of low and high affinity conformations, (2) intracellular signalling leads to LFA-1 clustering, (3) interaction with ligand stabilises the high affinity conformation of LFA-1. It has been difficult to distinguish between these three options. At present the model in **Figure 7.1 B** seems least likely because, as described in this thesis, most inside-out activators of LFA-1 adhesion cause clustering but not a detectable affinity alteration.

7.1.5 Integrin Dysfunction

Chapter 6 describes the characterisation of leukocytes from a patient who first presented with symptoms characteristic of Glanzmann's Thrombasthenia, a bleeding disorder due to mutation of the genes encoding the platelet integrin $\alpha_{\text{IIb}}\beta_3$. Subsequently, indications of Leukocyte Adhesion Deficiency-1 became apparent. This disease is due to mutations in the leukocyte integrin β_2 gene that prevent function and/or expression of LFA-1, Mac-1 and p150,95. Typically, LAD-1 patients have reduced emigration of leukocytes to inflammatory sites resulting in granulocytosis and susceptibility to bacterial infections. Leukocytes from this patient have normal levels of integrin expression. However, these integrins do not function properly. Inside-out stimuli failed to induce LFA-1-dependent T cell adhesion to ICAM-1 or ICAM-3 or Mac-1-mediated neutrophil adhesion to fibrinogen. Unexpectedly, $\alpha_4\beta_1$ -dependent T cell adhesion to VCAM-1 and $\alpha_4\beta_1$ - and $\alpha_5\beta_1$ -dependent T cell adhesion to fibronectin were also found to be affected. All these integrins did bind their ligands when stimulated with divalent cations or with activating mAbs. In addition, our collaborators found that $\alpha_{\text{IIb}}\beta_3$ mediated platelet adhesion to fibrinogen was defective. As at least three families of integrins could not be activated by intracellular signalling pathways, we concluded that this patient has a novel form of integrin dysfunction. We reasoned that the defect is unlikely to be in the integrin genes, but may be in a gene encoding a signalling protein or an integrin-associated protein required for activation.

7.2 FUTURE DIRECTIONS

In the immediate future, I will concentrate on further characterisation of leukocytes from the patient with integrin dysfunction. In cell of non-haematopoietic origin, integrins are constituents of complexes including multiple signalling molecules, cytoskeletal components and adapter molecules. These complexes are not well characterised in leukocytes. Identification of the lesion responsible for the integrin dysfunction could provide an insight into the molecular mechanisms responsible for signal transduction and integrin activation in haematopoietic cells. At the protein level, the initial approaches to identify this protein will be to run three dimensional gels to compare cell lysates from the patient and a control donor for any abnormal spots. Some clue to the identity of the defective protein may be gained from comparing the pattern of protein phosphorylation in lysates from activated cells from Patient FM and a control donor. If these methods do not yield any conclusive results, then the possibility of identifying differentially expressed genes could be explored. Either RNA from the patient could be used to generate probes to screen cDNA expression arrays, or differentially expressed transcripts could be enriched by subtractive hybridisation. Finally, expression cloning could be tried. In this technique an appropriate cDNA library, cloned into an expression vector, would be used to transfect cells from the patient and these cells would then be screened for restoration of integrin function. After several rounds of cloning the cDNA from the positive cells is isolated and the gene identified. However, transfecting leukocytes is problematic.

This patient also provides a unique opportunity to investigate physiological roles for affinity regulation of LFA-1. For example, it is reported that crosslinking CD31 increases the affinity of LFA-1 (Reedquist et al., 2000). It would be interesting to test whether this is possible with the patient's cells.

The differential effects of the β_2 mAbs on PdBu- and Mg^{2+} /EGTA-stimulated adhesion are worth further investigation. One likely possibility is that these mAbs block the clustering of LFA-1 that is required for PdBu-stimulated adhesion. Confocal microscopy could be used to determine whether this is the case. Peptides from the β_2 -I-like domain could be tested in a similar manner to the I domain peptides in Chapter 4.

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APPENDIX I

ONE AND THREE LETTER SYMBOLS FOR THE AMINO ACIDS

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lycine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The one letter symbol for an undetermined or non-standard amino acid is X

APPENDIX II

GMEM-S MEDIUM

Distilled water	400 ml	
Glasgow MEM	50 ml	Gibco-BRL
7.5% sodium bicarbonate	18.1 ml	Gibco-BRL
100 x MEM non-essential amino acids	5 ml	Gibco-BRL
100 x glutamine and asparagine	5 ml	see below
100 mM sodium pyruvate	5 ml	Gibco-BRL
50 x nucleosides	10 ml	see below
5000 units/ml penicillin-streptomycin	5 ml	Gibco-BRL
Dialysed FCS	to desired %	Gibco-BRL

100 x glutamine and asparagine

600 mg L-glutamic acid (Sigma) and 600 mg L-asparagine (Sigma) in 100 ml distilled water. Filter sterilised and stored at 4°C.

50 x nucleosides

35 mg each of adenosine, guanosine, cytidine and uridine and 12 mg of thymidine (all from Sigma) in 100 ml distilled water. Filter sterilised and stored at 4°C.

APPENDIX III

COMPANIES AND ADDRESSES

American Type Culture Collection

10801 University Boulevard, Manassas, VA, USA

Amersham Pharmacia Biotech. UK Ltd.

Amersham Place, Little Chalfont, Buckinghamshire, UK

Anachem Ltd.

Anachem House, Charles Street, Luton, Bedfordshire, UK

Becton Dickinson UK Ltd.

Between Towns Road, Oxford, UK

Bio-Rad Laboratories Ltd.

Bio-Rad House, Marylands Avenue, Hemel Hempstead, Hertfordshire, UK

Calbiochem

CN Biosciences UK, Boulevard Industrial Estate, Nottingham, UK

Cambridge Bioscience

24-25 Signet Court, Cambridge, UK

Chemicon International Ltd.

2 Admiral Way, Harrow, UK

Chiron UK Ltd.

Salamander Quay West, Harefield, Middlesex, UK

Corning Costar UK

1 The Valley Centre, Gordon Road, High Wycombe, Buckinghamshire, UK

Dako Ltd.

Denmark House, Angel Drove, Ely, Cambridge, UK

Dynex Technologies

Action Court, Ashford, Middlesex, UK

Eurogenetics UK Ltd.

Kingsway Business Park, Oldfield Road, Hampton, UK

Genetic Research Instrumentation Ltd.
Gene House, Queensborough Lane, Rayne, Essex, UK

Gibco-BRL
Life Technologies Ltd., Inchinnan Business Park, Paisley, UK

Jackson Immunoresearch Laboratories
UK distributor: Stratech Scientific Ltd., Dudley Street, Luton, Bedfordshire, UK

Millipore (UK) Ltd.
The Boulevard, Blackmoor Lane, Watford, Hertfordshire, UK

Molecular Probes
UK distributor: Cambridge Bioscience

Murex Biotech Ltd.
Central Road, Temple Hill, Dartford, UK

National Blood Service - South Thames
75 Cranmer Terrace, Tooting, London, UK

Nycomed
Oslo, Norway

PeproTech EC Ltd.
23 St. James' Square, London, UK

PerSeptive Biosystems
Birchwood Science Park North, Warrington, Cheshire, UK

Photon Technologies International
Suite 3, The Sanctuary, Oakhill Grove, Surbiton, Surrey, UK

Pierce
Pierce and Warriner (UK) Ltd., 44 Upper Northgate Street, Chester, Cheshire, UK

Santa Cruz Biotechnology Inc.
UK distributor: Autogen Bioclear UK Ltd., Holly Ditch Farm, Mile Elm, Calne, Wiltshire, UK

Serotec Ltd.
22 Bankside, Kidlington, Oxford, UK

Sigma
Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, UK

Scanalytics.

8550 Lee Highway, Fairfax, VA, USA

Southern Biotechnology Associates Inc.

UK distributor: Cambridge Bioscience

Titertek

UK distributor: Biological Instrumentation Services Ltd., Kirkham Trading Park,
Kirkham, Lancashire, UK

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BBA Molecular Cell Research in press

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